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CHARACTERIZATION OF NOTCH1 AND PI3K-PTEN-AKT/mTOR PATHWAY INTERACTION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

Kyriante' Henry

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**CHARACTERIZATION OF NOTCH1 AND PI3K-PTEN-AKT/mTOR PATHWAY
INTERACTION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA**

by

Kyriante' Savonn Henry, B.S.

APPROVED:

Faye M. Johnson, M.D., Ph.D.
Advisory Professor

Michael A. Davies, M.D., Ph.D.

Mitchell J. Frederick, Ph.D.

Gordon B. Mills, M.D., Ph.D.

Andrew J. Bean, Ph.D.

APPROVED:

Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

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INTERACTION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA**

A

THESIS

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center
UTHealth Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
of the Degree of

MASTER OF SCIENCE

By

Kyriante' Savonn Henry, B.S.

Houston, Texas

December 2017

Dedication

First and foremost, I would like to thank my Lord and Savior, Jesus Christ, for blessing me with the opportunity to complete my scientific studies at such a prestigious institution as The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences. Though the journey was challenging and arduous, I am proud to say that I successfully completed my thesis project because of the grace and mercy of my Heavenly Father. After all, “I can do all things through Christ who strengthens me (Philippians 4:13).”

This thesis is dedicated to all of the people who have loved and encouraged me as I worked to complete my thesis project during the past two years, and those who continue to support me as I embark on future endeavors in my scientific career.

To my grandparents, Nell Duncan, Alice Duncan, Mary Henry, Louis Henry, Jr., Wayne Bartholomew, and Lloyd Bourgeois: I thank each of you for nurturing me with the love, care, and guidance that I needed to believe in myself and become who I was destined to be.

To my mother, Kione Duncan: Thank you for giving me life and being the push that I needed to persevere. You mean more to me than words can express.

To my siblings, Kodie, Koby, Tyler, Lil Man, and Imani: Thank you for holding me accountable for every word, deed, and action. Being the oldest of you all, I pray that I continue to be an exemplar role model for each and every one of you. Through all of the trials and tribulations that life may bring, you must stop at nothing to achieve your dreams and accomplish your goals. It is through discipline, hard work, perseverance, and confidence in yourself that you will prevail. When the road gets that much tougher, know that I'll be there to help guide each of you along the way.

To my step-mother, Cocoa Green, and my godfather, Odel McKnight: Thank you both for always being there to cheer me along and always being there for me when I needed you. I am forever grateful to the both of you.

To my boyfriend, Walter Peterson, Jr.: Thank you for being my support system, always there to encourage and uplift me when I needed it most. Thank you for every clean dish, every folded article of laundry, and every cooked meal. Thank you for taking care of my well-being when I was too tired and frustrated from the lab to take care of myself.

To my guardian Angels, A'nara, LaTanya, and Mary Henry: Thank you for constantly guiding me, watching over me, and protecting me. I know that you all are fighting the battles for me that I cannot fight for myself. I feel your presence all the time and I pray that I am making you proud. Though it took you all from this life too soon, I promise you that one day very soon we will eradicate the menace that is cancer. This thesis is a genuine dedication to you all!

Last, but certainly not least, to my twin, my backbone, my Daddy, Louis Henry, Sr.: Words cannot even begin to express how much you truly mean to me. Thank you for being my biggest critic and my biggest cheerleader, always giving me the strength and confidence that I needed to keep going when I was ready to give up. With your unconditional love and guidance, I know that I can achieve the unthinkable. I am who I am because of who you are. Thank you for instilling in your Princess the morals and values that I needed to succeed in this world. You are truly a blessing to my life, the best father a daughter can ask for. This is only the beginning of the Henry Empire. TEAM HENRY FOREVER!

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CHARACTERIZATION OF NOTCH1 AND PI3K-PTEN-AKT/mTOR PATHWAY INTERACTION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

Kyriante' Savonn Henry, B.S.

Advisory Professor: Faye M. Johnson, M.D., Ph.D.

Head and neck squamous cell carcinoma (HNSCC) affects various mucosal sites of the upper aerodigestive tract, including the nasal and oral cavities, the nasopharynx, and the oropharynx. More than five hundred thousand new cases of HNSCC occurred in 2011 alone, with 50,000 reported cases in the United States. This trend made HNSCC the seventh most common non-skin cancer worldwide (Ferlay et al., 2015). Although significant epidemiological and pathological advancements have been made, survival rates have not improved much over the last 40 years, leaving a mortality rate that remains at approximately 50%. An unbiased drug screen demonstrated that HNSCC cell lines bearing *NOTCH1* inactivating mutations are sensitive to PI3K and dual PI3K/mTOR inhibitors, leading us to investigate the interaction of these pathways in HNSCC. The NOTCH pathway plays key roles in cell proliferation, differentiation, and apoptosis. With inactivating mutations in 18% of 510 HNSCC patient samples, *NOTCH1* is one of the most frequently altered genes in HNSCC (TCGA, Provisional). In 21 patients with HNSCC tumors, approximately 28 *NOTCH1* mutations predicted tumor suppressive properties (Agrawal et al., 2011). The PI3K/mTOR pathway is a critical regulator of cell growth, proliferation, differentiation, and survival. It is the most frequently altered pathway in HNSCC, occurring in approximately 80% of HNSCC tumors (Iglesias-Bartolome R, et al., 2013). Previous research suggests that NOTCH1 regulates PI3K-

AKT and mTOR1 signaling in T-ALL cells by decreasing PTEN expression. In addition, blocking NOTCH1 activity has been shown to upregulate PTEN activity in T-ALL cells via Hes1 downregulation (Palomero T, et al., 2007). Unfortunately, the mechanism of communication between the two pathways is unknown in HNSCC. Understanding the interaction between the NOTCH1 and PI3K/AKT/mTOR signaling pathways presents them as essential targeting agents that may have a significant impact on modern therapeutic medicine. In this research, we sought to investigate whether the NOTCH1 pathway regulates the activity of the PI3K-PTEN-AKT/mTOR pathway in HNSCC *in vitro*. The data of various experiments aimed at manipulating NOTCH1 activation and HES1 expression suggests that the NOTCH1 pathway does not affect the PI3K-PTEN-AKT/mTOR pathway in HNSCC.

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CHAPTER 1: INTRODUCTION

1.1 Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinoma (HNSCC) ranks among the most lethal, common non-skin types of cancer around the world. The disease affects ~600,000 people around the world annually (Jemal et al., 2011). More than five hundred thousand new cases of HNSCC occurred in 2011 alone, with 50,000 reported cases in the United States (Agrawal et al., 2011). These trends make HNSCC the seventh most common non-skin cancer worldwide (Ferlay et al., 2015). HNSCC affects various mucosal sites of the upper aerodigestive tract, including the nasal cavity, the oral cavity, the larynx, and the pharynx. Figure 1 illustrates the regions of the upper aerodigestive tract that are affected by head and neck cancer. Tobacco use, alcohol consumption, and infection with high-risk types of human papillomavirus (HPV) are major risk factors for developing HNSCC (Stransky et al., 2011). In fact, the combination of tobacco and heavy alcohol consumption has a strong synergistic effect on worsening disease prognosis. The 5-year survival rate is only ~50% in head and neck cancer (Jemal et al., 2011; Kamangar et al., 2006). Although previous research has made significant epidemiological and pathological advancements, survival rates have not improved much over the last 40 years (Agrawal et al., 2011). Some treatment methods can result in various cosmetic deformities and impaired vital functions, such as difficulty breathing, swallowing, speaking, tasting, hearing, and smelling. Cetuximab, a monoclonal antibody targeting epidermal growth factor receptor (EGFR), is currently the only approved targeted therapy for treating head and neck cancer. However, only a subtle improvement has been detected in the overall survival of head and neck cancer patients (Baselga et al., 2005). A deeper understanding of this morbid malignancy's pathogenesis and the role of recently identified genetic mutations is needed to develop more effective and targeted therapeutic approaches.

Head and Neck Cancer Regions

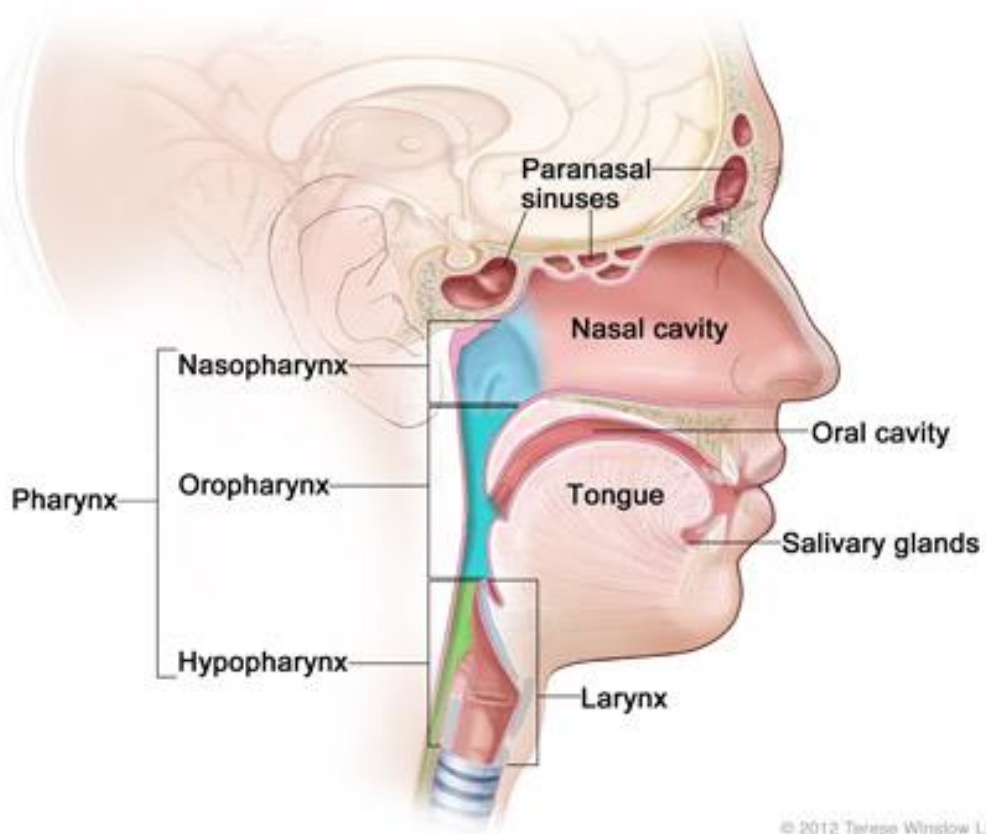


Figure 1: Anatomy of Head and Neck Squamous Cell Carcinoma.

Head and neck squamous cell carcinoma affects various mucosal sites of the upper aerodigestive tract. This encompasses the nasal cavity, the oral cavity, the paranasal sinuses, the tongue, the salivary glands, the larynx, and the pharynx (including the nasopharynx, the oropharynx, and the hypopharynx). *Permission obtained from Argiris, A., Karamouzis, M.V., Raben, D., Ferris, R.L. (2008). Lancet. 371, 1695-1709. (Argiris et al., 2008).*

1.2 PI3K-PTEN-AKT/mTOR pathway

The phosphoinositide-3-kinase (PI3K) pathway is perhaps the most frequently activated signaling pathway in human cancer. It plays a key role in the regulation of multiple cellular events, including cell growth, proliferation, cell cycle progression, and survival (Vivanco and Sawyers, 2002). PI3Ks phosphorylate the 3'-hydroxyl group of phosphatidylinositides. The PI3K family of enzymes is divided into three main classes (classes I-III), based on the structure and substrate specificity of each of the enzymes. Class I PI3Ks are those most often implicated in a wide array of human cancers (Engelman, 2009). In mammals, class I PI3Ks are further divided into subclasses, IA and IB, based on their modes of regulation. Class IA PI3Ks are heterodimers that comprise a regulatory subunit (p85), which mediates binding of the enzymatic ligand to membrane growth factor receptors, and one of four catalytic subunits (p110 α , - β , - γ , or - δ), which are responsible for the activity of the enzyme (Engelman, 2009). The genes *PIK3CA*, *PIK3CB*, and *PIK3CD* encode the three highly homologous class IA catalytic isoforms: p110 α , p110 β , and p110 δ , respectively. These isoforms associate with any of the five regulatory isoforms: p85 α (and its splicing variants, p55 α and p50 α), p85 β , and p55 γ , which are collectively called p85-type regulatory subunits (Engelman et al., 2006). Class IB PI3Ks are heterodimers of a p110 γ catalytic subunit, encoded by *PIK3CG*, coupled with the regulatory isoforms, p101 or p87 (Liu et al., 2009). Whereas p110 α and p110 β are ubiquitously expressed, p110 δ and p110 γ expression are generally restricted to leukocytes (Okkenhaug and Vanhaesebroeck, 2003).

Activated by autocrine signaling, the PI3K/mTOR pathway plays a critical role in cancer cell activation and provides self-sustaining growth signals to tumors. The pathway can also be activated by paracrine signaling. In the absence of activating signals, p85 interacts with p110 to inhibit p110 kinase activity. Following receptor tyrosine kinase

(RTK) or G protein-coupled receptor (GPCR) activation, class I PI3Ks are recruited to the plasma membrane, where p85-mediated inhibition of p110 is relieved and p110 phosphorylates phosphatidylinositides 4,5-bisphosphate (PIP₂) to generate phosphatidylinositides 3,4,5-trisphosphate (PIP₃). The lipid product, PIP₃, acts as a second messenger of the pathway. It can activate AKT-dependent and AKT-independent downstream signaling pathways (Vanhaesebroeck et al., 2010).

AKT (protein kinase B) is a serine/threonine kinase expressed as three isoforms: AKT1, AKT2, and AKT3. All three isoforms share a similar structure: an N-terminal PH domain, a central serine/threonine catalytic domain, and a small C-terminal regulatory domain (Lu et al., 2012). Activation of AKT is initiated by its translocation to the plasma membrane, where the PH domain in the N-terminal region of the kinase docks to PIP₃. This results in a conformational change in AKT, exposing two amino acid residues that are critical to the phosphorylation and activation of AKT. The full activation of AKT requires the phosphorylation of threonine 308 (Thr308) by *PDPK1* (phosphoinositide-dependent protein kinase 1) and the phosphorylation of serine 473 (S473) by *PDPK2* (phosphoinositide-dependent protein kinase 2) (Velichkova et al., 2010). Under most circumstances, mTORC2 is the primary source of *PDPK2*. Once activated, AKT phosphorylates various other proteins, including GSK3 (glycogen synthase kinase 3) and FOXOs (the forkhead family of transcription factors). Thus, AKT regulates a wide array of cellular processes involved in protein synthesis, cell survival, cell proliferation, and metabolism (Parsons, 2004).

mTOR (mammalian target of rapamycin) belongs to a group of serine/threonine protein kinases that are referred to as class IV PI3Ks. The kinase serves as a pivotal regulator of cell growth, cell survival, and cell proliferation by monitoring nutrient availability, cellular energy and oxygen levels, and mitogenic signals (Song et al., 2012).

mTOR also plays a key role in tumorigenesis. mTOR exists in two structurally distinct complexes: mTORC1 and mTORC2. The mTORC1 complex is composed of the mTOR catalytic subunit, Raptor (regulatory associated protein of mTOR), PRAS40 (proline-rich AKT substrate 40 kDa), and the protein mLST8/GbL. mTORC2 is composed of mTOR, Rictor (rapamycin insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein 1), and mLST8/GbL (Chang et al., 1997). While mTORC1 controls protein synthesis by activating translation of proteins, mTORC2 affects metabolism and cell survival by fully phosphorylating and thus activating AKT. However, mTORC2 is less defined than mTORC1.

Partial AKT activation is sufficient to activate mTOR. AKT activates mTOR by directly phosphorylating tuberous sclerosis complex 2 (TSC2) to attenuate its inhibitory effects on mTOR1 (Klippel et al., 1996). The tuberous sclerosis complex (TSC), an essential negative regulator of mTORC1 activity, comprises tuberous sclerosis complex 1 (TSC1; hamartin) and tuberous sclerosis complex 2 (TSC2; tuberin). TSC2 functions as a GTPase-activating protein (GAP) for Rheb. The active, GTP-bound form of Rheb directly interacts with mTORC1 to stimulate its activity. As a Rheb-specific GAP, TSC2 negatively regulates mTORC1 signaling by converting Rheb into its inactive GDP-bound state. TSC1 does not have a GAP domain, but it acts as a stabilizer of TSC2 by protecting it from degradation (Klippel et al., 1996). Hence, the TSC complex functions as a tumor suppressor.

The best characterized downstream targets of mTORC1 are S6K1 (p70S6 kinase) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1). Both effectors are critically involved in regulating protein synthesis and protein translation, respectively. S6K1 is a serine/threonine kinase that belongs to the family of AGC kinases. It is responsible for phosphorylating its core substrate, rpS6 (ribosomal protein

S6), resulting in cell growth and cell size regulation. While the activating phosphorylation of rpS6 at serines 240 and 244 (S240/244) are mediated by the PI3K/mTOR pathway, the activating phosphorylation of rpS6 at serines 235 and 236 (S235/236) are mediated by MEK/ERK pathway (Meyuhas, 2008). 4EBP1 is a member of a family of translation repressor proteins. It prevents translation initiation by binding to the translation factor, eIF4E. The interaction of hypo-phosphorylated 4EBP1 and eIF4E prevents its interaction with eIF4G, thereby blocking complex assembly and repressing translation. Inhibitory phosphorylation of 4EBP1 at serine 65 (S65) by mTORC1 causes its release from eIF4E, allowing cap-dependent translation to proceed (Ma et al., 2009). Thus, the activation of mTOR provides tumor cells with a growth advantage by promoting protein synthesis.

The primary negative regulator of the PI3K pathway is the phosphatase and tensin homolog (PTEN). It negatively regulates the PI3K/mTOR pathway by removing the 3-phosphate from PIP₃, converting it back to PIP₂ (Chia et al., 2010). PTEN is a lipid phosphatase that attenuates the proliferative phenotype of various types of cancer cells by antagonizing the activity of PI3K. Thus, PTEN serves as an essential tumor suppressor. Although PTEN is a highly stable protein, it is tightly regulated at the transcriptional level and is often regulated by post-translational modifications. While methylation of the promote region inactivates the *PTEN* gene, phosphorylation of PTEN at amino acid residues, serine 380 as well as threonines 382 and 383, inhibits protein activity (Salmena et al., 2008). A simplified illustration of the PI3K-PTEN-mTOR pathway is depicted in Figure 2.

Genetic alterations in the PI3K pathway have been associated with a great deal of cancers. Hyperactivity of the PI3K pathway is one of the most prominent characteristics of many human tumors. Current research reports that pathway activity is upregulated in 30-50% of prostate cancers (De Velasco and Uemura, 2012; Suzuki et

al., 1998). Class IA PI3Ks are mutated and amplified in a wide array of cancer types. The *PIK3CA* gene encodes the p110 α catalytic subunit of PI3K. It was found to be frequently mutated in 27% of breast cancers, 23% of endometrial cancers, 14% of colorectal cancers, 17% of urinary tract cancers, and 8% of ovarian cancers (Samuels et al., 2004). Most frequently activated mutations (also called “hot-spots”) in PI3K are located within the kinase domain, H1047, and the helical domains, E542 and E545 (Samuels et al., 2004). 4-10% of esophageal squamous cell carcinomas exhibit activating mutations of *PIK3CA*. Mutations within the E542K and E545K hot-spots and the H1047R substitutions were the most common (Song et al., 2014). Furthermore, 40% of esophageal tumors present an increase in *PIK3CA* copy number, a feature that is associated with poor disease prognosis (Akagi et al., 2014; Lin et al., 2014; Song et al., 2014).

One common mechanism promoting aberrant PI3K signaling is the somatic ablation of *PTEN* by genetic and epigenetic modifications. The *PTEN* gene maps to the human chromosome 10q23.3, a region that displays high rates of heterozygosity loss in various cancer types, including kidney, lung, breast, and prostate cancer (Kwabi-Addo et al., 2001). Previous research suggests that in aggressive forms of prostate cancer, the *PTEN* gene is subject to DNA sequence alterations and point mutations that inhibit the activity of the PTEN protein (Dong, 2006). Ablation of *PTEN* often results in unrestrained signaling of the PI3K pathway, which may lead to tumorigenesis. In fact, loss of *PTEN* in most cancer types leads to hyper-activation of AKT, which is associated with uncontrollable cell proliferation, decreased apoptosis, and enhanced tumor angiogenesis (Carnero et al., 2008). Taken together, the findings suggest that the loss of the tumor suppressive function of *PTEN* is critically linked to tumorigenesis and cancer progression.

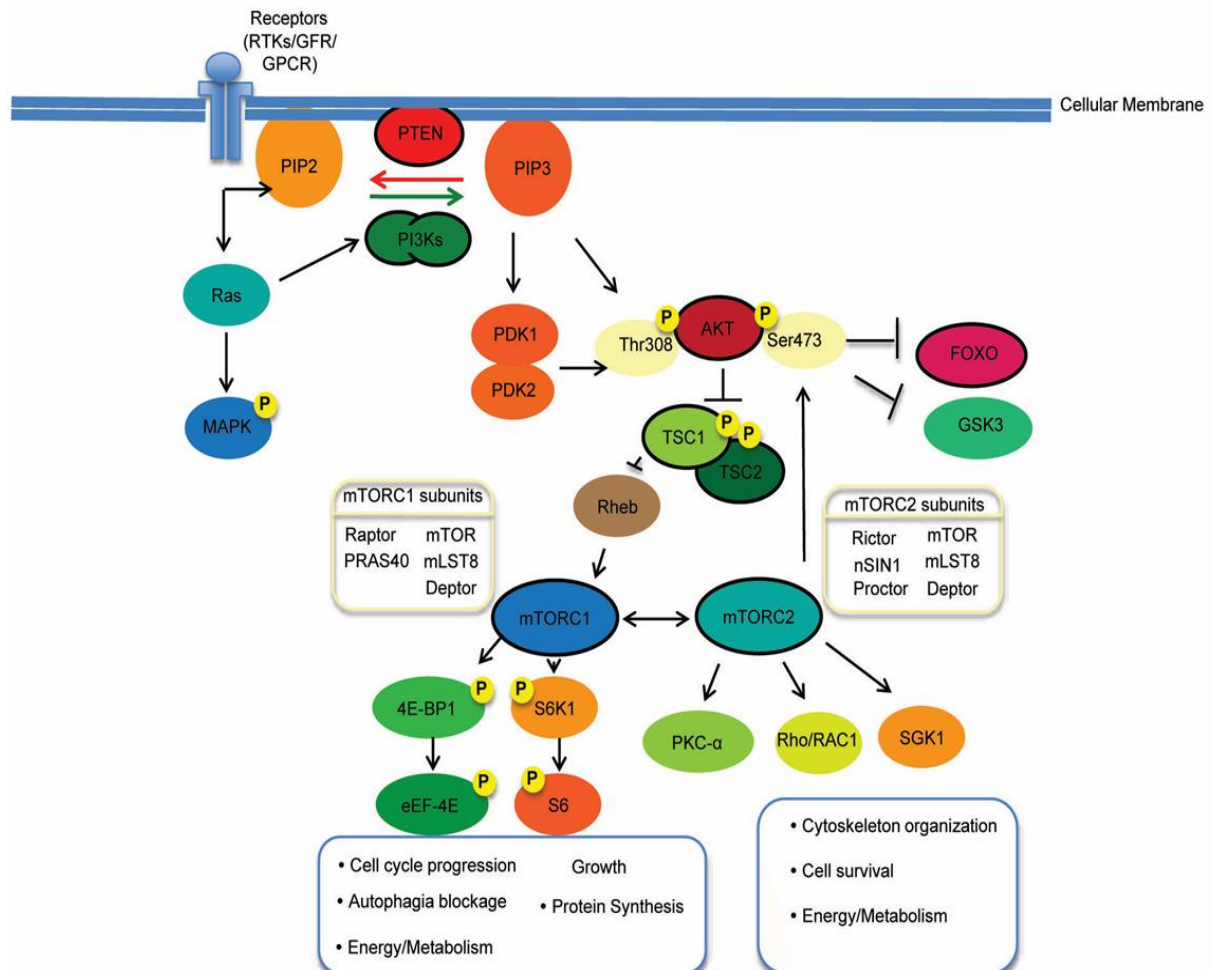


Figure 2: The PI3K-PTEN-AKT/mTOR pathway.

Activation of the PI3K/mTOR pathway via endogenous stimuli, exogenous signals, or mutations promotes cell growth, proliferation, cell cycle progression, and survival. The PI3Ks are a complex of heterodimers that encompass regulatory and catalytic subunits. Upstream cellular receptors (such as RTKs and GPCR) promote the activation of the PI3Ks, which initiates a downstream signaling cascade via phosphorylation. The PI3Ks phosphorylate the membrane phospholipid substrate, PIP₂, converting it into PIP₃. PTEN is a phosphatase that reverts the activity of PI3K by removing the 3-phosphate from PIP₃, converting it back to PIP₂. Therefore, an inactivating mutation or loss of PTEN leads to hyper-activation of the PI3K/mTOR pathway. PIP₃ then recruits AKT to the plasma membrane, where PDK1 and PDK2 fully activate AKT by phosphorylating Thr308 and S473, respectively. Once activated, AKT phosphorylates and inhibits the TSC1/2, leading to the activation of the mTOR complex (mTORC1 and mTORC2). Activated mTORC1 phosphorylates downstream effectors, S6K1 and 4EBP1. Thus, the PI3K-PTEN-mTOR pathway can control essential cancer cell processes, including proliferation, invasion, metastasis, and response to therapy. *Permission obtained from Giudice, F., and Squarize, C. (2013). J Carcinogene Mutagene. S5: 003. (Giudice et al., 2013).*

1.3 PI3K-PTEN-AKT/mTOR pathway in HNSCC

The PI3K/AKT/mTOR pathway plays a key role in regulating several cellular processes in normal and cancer cells, including proliferation, growth, invasion, migration, and survival. Currently, the PI3K/AKT/mTOR is the most frequently altered pathway in HNSCC. Approximately 80% of HNSCC tumors contain genetic alterations in one or more components of the pathway (Iglesias-Bartolome et al., 2013; Lui et al., 2013). According to the most current data produced by the head and neck cohort of The Cancer Genome Atlas (TCGA), 18% of 510 HNSCC tumors exhibit mutations in the *PIK3CA* gene (Gao et al., 2013). The frequency of the pathway alterations promotes poor disease prognosis.

In HNSCC, particularly, the type and frequency of genetic alterations in the PI3K/mTOR pathway vary immensely. Many of the alterations occur in the *PIK3CA* gene. Findings of recent research report gain-of-function mutations in *PIK3CA* in 6-20% of tumors. Other studies report *PIK3CA* overexpression in 52% of tumors and *PIK3CA* amplification in 30% of tumors. The hot-spot substitutions, E542K, E545K, and H1047R, were the most common (Agrawal et al., 2011; Stransky et al., 2011; Suda et al., 2012; Lui et al., 2013). Some of the alterations also occur in PTEN, both at the gene and protein levels. Previous research findings report loss of heterozygosity (LOH) of *PTEN* in 8% of tumors and inactivating *PTEN* mutations in 4% of tumors. Likewise, reduced PTEN protein expression occurred in 30% of tumors (Squarize et al., 2013; Morris et al., 2011). There were also mutations in the *PIK3CG* gene in 4% of tumors (Agrawal et al., 2011; Stransky et al., 2011). Furthermore, while mutations in the *AKT* and *mTOR* genes occur rarely, phosphorylation of AKT and S6 at the protein level are frequently exhibited in almost all HNSCC tissues (Lui et al., 2013; Molinolo et al., 2007). This phosphorylation is an indication of constitutive pathway activation.

1.4 PI3K/mTOR pathway inhibition in HNSCC

The frequent activation of the PI3K/mTOR pathway in HNSCC and the pathway's significant effect of cancer cell signaling make it a promising target for desperately needed improvements in various cancer therapies. Because the pathway's activation is regulated by identifiable genetic alterations, it is important to use suitable biomarkers for predicting response to those therapies. For these reasons, many pharmaceutical companies and academic laboratories are actively developing inhibitors to target the entire pathway and its key components. Wortmannin and LY294002 are two well-known, first generation PI3K inhibitors. Wortmannin binds irreversibly binds to PI3K enzymes by covalently modifying a lysine necessary for catalytic activity, while LY294002 is a small-molecule inhibitor that reversibly targets the PI3K family members (Liu et al., 2009). Unfortunately, both inhibitors have miniscule selectivity for the distinct isoforms of PI3K and are rather toxic in animals (Knight and Shokat, 2007). Despite their limitations, the preclinical studies influenced by the inhibitors have greatly contributed to our understanding of the biological significance of the PI3K pathway.

Several compounds targeting PI3K activity have been introduced into clinical trials, with many of them being dual PI3K/mTOR inhibitors. BEZ235 inhibits multiple class I PI3K isoforms and mTOR kinase activity by binding to the ATP-binding pocket (Maira et al., 2008). Preclinical data suggests that it displays strong anti-proliferative properties on tumor xenografts that exhibit many key genetic alterations in the PI3K pathway, including PTEN ablation and gain-of-function PI3K mutations (Serra et al., 2008). BEZ235 has entered Phase I clinical trials in patients with solid tumors (Garcia-Echeverria and Sellers, 2008). BGT226 and BKM120, a selective class I PI3K inhibitor with no inhibitor effects on mTOR activity, have also entered Phase I clinical trials (Knight and Shokat, 2007).

Previous studies report that head and neck tumors with activating alterations in *PIK3CA* respond better to targeted therapies with specific PI3K α inhibitors (Elkabets et al., 2013; Mazumdar et al., 2014). Of note, the first-in-human clinical trial of BYL719, a specific PI3K α inhibitor, in solid tumors (NCT01387321) reported that eight patients with head and neck tumors harboring *PIK3CA* mutations had a clinical response to therapy (Baselga et al., 2014, Annals of Oncology, abstract). Despite the frequent activation of the PI3K/mTOR pathway in HNSCC, using various chemical inhibitors to attenuate pathway activity has resulted in variable efficacy *in vitro* and *in vivo*.

1.5 NOTCH pathway

The NOTCH signal transduction pathway assumes numerous roles in various developmental processes. NOTCH signaling serves as a critical determinant of cellular fate within a vast array of tissues. The pathway regulates cell growth, differentiation, and apoptosis (Artavanis-Tsakonas et al., 1999). Activated by juxtacrine signaling, the interaction between the receptor of the signal-receiving cell and the ligand of the signal-sending cell activates the signaling cascade of the NOTCH pathway. There are four mammalian NOTCH receptors (NOTCH1-4). The receptors are evolutionarily conserved single-pass, heterodimeric transmembrane proteins composed of an extracellular (NECD) domain, a transmembrane (NTM) domain, and a NOTCH1 intracellular (NICD) domain. Additionally, there are five ligands: Delta-like (Dll-1, Dll-3, and Dll-4) and Jagged (Jagged-1 and Jagged-2), all of which are expressed on adjacent cells (Hansson et al., 2004). Figure 3 depicts the canonical NOTCH pathway.

Upon activation of the pathway, the ligand of the neighboring cell binds to the receptor of the NOTCH pathway, initiating a signaling cascade within the pathway. In the signal-receiving cell, NOTCH receptors are processed in the endoplasmic reticulum and

the Golgi apparatus via glycosylation and the first proteolytic cleavage, generating a calcium-stabilized heterodimer. The heterodimer, which consists of the NECD that is non-covalently attached to the TM-NICD complex, is transported to the plasma membrane of the cell by endosomes (Weinmaster, 1997). The receptor then undergoes two subsequent ligand-dependent proteolytic cleavages events. The second proteolytic cleavage involves tumor necrosis factor- α -converting enzyme (TACE), a transmembrane protein that includes an extracellular zinc-dependent protease domain. TACE cleaves the extracellular site of the NOTCH receptor between amino acid residues, alanine 1710 and valine 1711, leaving the NECD bound to the ligand of the signal-sending cell (Mumm and Kopan, 2000).

The third proteolytic cleavage involves γ -secretase, an integral membrane protein that cleaves transmembrane proteins at amino acid residues within the transmembrane domain. The γ -secretase complex consists of presenilin, nicastrin, Pen-2, and Aph-1. The γ -secretase complex cleaves the intracellular region of the receptor, releasing the active form of NICD (Bray, 2006). NICD is then released into the cytoplasm, where it is subsequently translocated into the nucleus to establish NOTCH signaling and begin transcription. In the absence of NICD from the nucleus, the NOTCH target gene expression is inactivated by a complex referred to as "CSL." The complex encompasses a transcriptional repressor protein called C protein binding factor 1 (CBF1), Suppressor of hairless [Su(H)], and Lag1. Once NICD is present in the nucleus, it binds to the CSL complex, converting CBF1 from a transcriptional repressor to a transcriptional activator and displacing the other co-repressors (Weinmaster, 1997).

The CSL-NICD complex then recruits a co-activator complex that contains Mastermind (MAML) and p300. Mastermind (MAML) is a 3-member family of transcriptional activator proteins. p300 is a histone acetyltransferase that epigenetically

modifies that structure of chromatin to a form that is amenable to active transcription. Once the complexes bind efficiently in the nucleus, the NOTCH pathway is fully activated and transcription takes place (Wu et al., 2000). The NOTCH target genes include AKT, mTOR, and HES/HEY family members. AKT and mTOR are key components of the PI3K/mTOR pathway.

HES1 (hairy enhancer of split-1) is one of ten members (HES1-7; HEY1, HEY2, HEYL) of the HES and HEY families. *HES* and *HEY* genes encode nuclear proteins that suppress transcription. Like HEY1 (hairy enhancer-of-split related with YRPW motif protein 1), HES1 belongs to the basic helix-loop-helix (bHLH) families of transcription factors (Leimeister et al., 1999). It represses the transcription of genes that require the function of a bHLH protein for their transcription. The HES1 protein has a certain type of domain that contains a helix interrupting protein that binds to the N-box promoter region of the respective gene, rather than the canonical enhancer box (E-box) (Kageyama et al., 2007). As a member of the bHLH family, HES1 is a transcriptional repressor that regulates many cellular processes that influence development. It plays a pivotal role in cell proliferation and differentiation in embryogenesis (Kageyama et al., 2008).

The NOTCH1 pathway is complex because it can assume different roles in different cancer types. NOTCH1 can act as an oncogene or a tumor suppressor, depending on the context of the cancer. It is well-documented in the literature NOTCH1 functions as an oncogene in various types of leukemias and lymphomas. NOTCH1 was first identified as an oncogene in T-cell acute lymphoblastic leukemia, where activating *NOTCH1* mutations in T-cell acute lymphoblastic leukemia (T-ALL) led to ligand-independent NOTCH1 signaling (Weng et al., 2004). NOTCH1 exhibits oncogenic signaling in approximately 60% of human T-ALL tumors (South et al., 2012). The same role was later established in other B cell malignancies, such as chronic lymphocytic

leukemia (CLL) and mantle cell lymphoma (MCL), and breast adenocarcinomas. While *NOTCH1* functions as an oncogene in 5-12% of CLL tumors and approximately 10% of MCL tumors, it functions as an oncogene in less than 5% of breast adenocarcinomas (South et al., 2012).

NOTCH1 serves as a tumor suppressor in many different types of squamous cell carcinomas (SCCs). The tumor suppressor role of NOTCH1 signaling was first reported by the Radtke group. Their research showed that conditional knock out of NOTCH1 increased tumor incidence in squamous cell skin carcinomas (Radtke and Raj, 2003). Currently, active NOTCH1 signaling exerts tumor suppressive properties on 60-70% of squamous cell skin carcinomas. Additionally, NOTCH1 functions as a tumor suppressor in 5-10% of lung squamous cell carcinoma tumors (South et al., 2012). The diverse function of NOTCH1 in the context of a cancer cell renders it a novel targeting agent for improving modern cancer therapies.

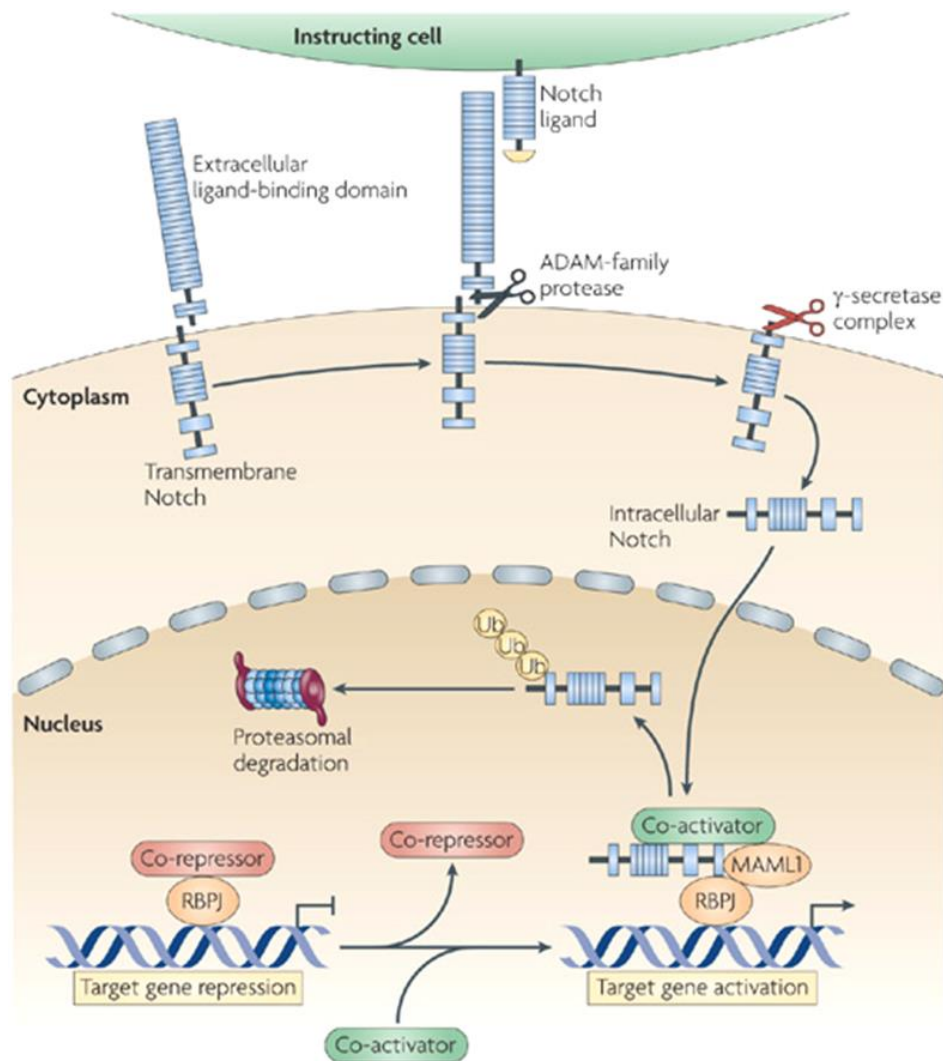


Figure 3: The NOTCH pathway.

The NOTCH pathway plays a key role in regulating cell fate determination, cell differentiation, and apoptosis. Upon activation, the intracellular domain of the NOTCH1 receptor (NICD) is cleaved. NICD then enters the cytoplasm and is translocated to the nucleus, where it aids in the transcription of various target genes. *Permission obtained from Amsen, D., Antov, A., Flavell, R. A. (2009). Nature Reviews Immunology. 9, 116-124. (Amsen et al., 2009).*

1.6 NOTCH1 pathway in HNSCC

Understanding the NOTCH1 pathway is essential to developing targeted therapies for head and neck squamous cell carcinoma. Multiple groups have performed whole exome sequencing on head and neck tumors and discovered frequent *NOTCH1* mutations in HNSCC (Agrawal et al., 2011; Stransky et al., 2011; Pickering et al., 2013). These findings were confirmed by the most current data produced by the head and neck cohort of The Cancer Genome Atlas (TCGA), in which *NOTCH1* mutations have a prevalence of approximately 18% in 510 HNSCC patient tumor samples (Gao et al., 2013). Of note, the majority of mutations are predicted to be inactivating *NOTCH1* mutations. Findings of recent research identified a pattern of truncating *NOTCH1* mutations that occurred frequently in HNSCC. Particularly, 28 *NOTCH1* mutations in 21 patients with HNSCC tumors predicted tumor suppressive properties (Agrawal et al., 2011).

More specifically, the oncogenic *NOTCH1* mutations exhibited by T-ALL occur in two locations along the gene. The first hot-spot consists of generally missense mutations within the negative regulatory heterodimerization domain. The second hot-spot consists of frequent truncating mutations near the C-terminus, leading to a deletion in the PEST domain and causing a markedly increased stabilization of constitutively activated NOTCH1 in the nucleus (Ferrando, 2009). In HNSCC, however, the distribution of inactivating *NOTCH1* mutations is completely different from the activating *NOTCH1* mutations found in T-ALL. There are some truncating mutations dispersed throughout the gene, but they are not located in the PEST domain. Most of the missense mutations cluster in the extracellular EGF-like binding domain, thereby preventing the NOTCH1 receptor from binding to the ligand and blocking NOTCH1 signaling (Pickering et al., 2014). The findings suggest that the *NOTCH1* mutations in HNSCC are loss-of-function

mutations. Figure 4 is an illustration of the locations of the mutations in T-ALL and HNSCC along the *NOTCH1* gene.

The results of the next generation sequencing analysis on HNSCC tumors and the mutational landscaping of *NOTCH1* in both cancer types support the hypothesis that NOTCH1 functions as a tumor suppressor in head and neck squamous cell carcinoma, unlike its well-established role as an oncogene in T-ALL. Researchers subsequently demonstrated that restoring NOTCH1 receptors with active NOTCH1 signaling to *NOTCH1* mutant HNSCC cell lines inhibited their *in vitro* growth on external ligands as well as their *in vivo* growth in mice (Pickering et al., 2013). Currently, inactivating NOTCH1 mutations have been discovered in a vast number of solid tumors derived from skin, lung, and esophageal squamous cell carcinomas (Pickering et al., 2014; group Tw, 2012; Agrawal et al., 2012). Taken together, the findings support the observation that *NOTCH1* is one of the most frequently occurring and commonly mutated novel genes in HNSCC (Agrawal et al., 2011).

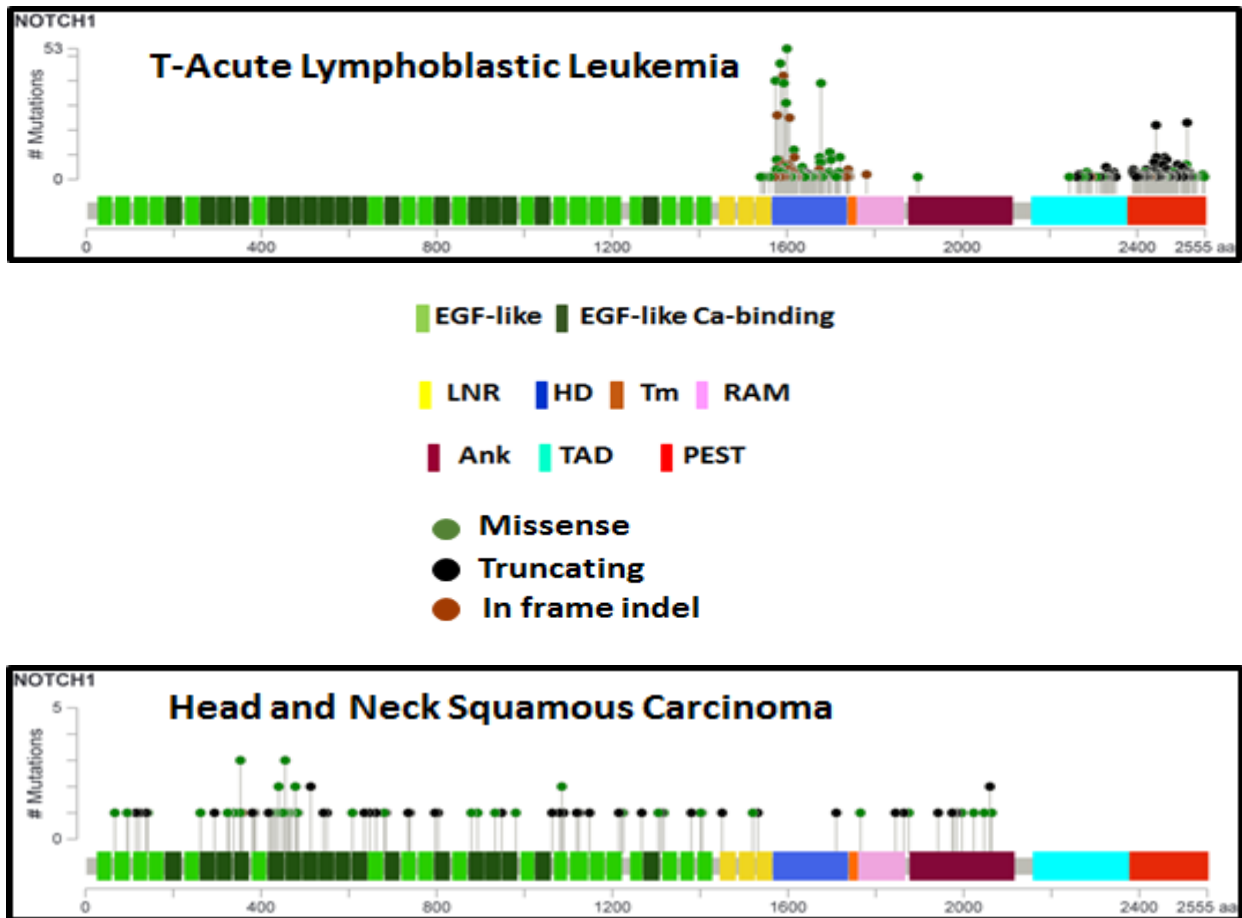


Figure 4: Genetic locations of *NOTCH1* mutations in T-ALL and HNSCC.

There are different patterns of *NOTCH1* mutations in T-ALL and HNSCC. Missense, truncating, and in-frame indel mutations are indicated with green, black, and brown dots, respectively. The *NOTCH1* domains are color-coded, with the heterodimerization (HD) and Lin-*NOTCH* repeat (LNR) domains constituting negative regulatory regions (Pickering et al., 2014; Figure: Mitchell J. Frederick, PhD).

1.7 Sensitivity of *NOTCH1* mutants to PI3K/mTOR inhibitors

To identify novel, translationally applicable molecular vulnerabilities to PI3K/mTOR pathway inhibitors, our laboratory used a pharmacogenomics approach to integrate drug sensitivity data for seven diverse PI3K/mTOR pathway inhibitors with multiple 'omic' data on a panel of molecularly characterized HNSCC cell lines. The association between drug response and molecular characteristics in the respective cell lines was studied. Sixty-eight head and neck squamous cell carcinoma cell lines were analyzed. They were stratified into 3 groups, based on their genotype. The cell lines were treated with 2 dual PI3K/mTOR inhibitors, exclusively, and cell viability was measured. Based on the best dose-response model, IC 70 values were generated for each cell line.

The same cell lines were also treated with selective class I PI3K and pan-PI3K inhibitors. The drug screening experiments in the laboratory revealed that the cell lines that harbored *NOTCH1* and *PIK3CA* mutations were more sensitive to the selective class I PI3K, the pan-PI3K, and the dual PI3K/mTOR inhibitors than their wild-type counterparts. Drug-induced apoptosis was also measured. The cell lines treated with dual PI3K/mTOR inhibitors exhibited significant increases in cell death. The results, however, were irrespective of the *PIK3CA* status of the cell lines. *NOTCH1* mutant HNSCC cells lines were sensitive to dual PI3K/mTOR inhibitors and underwent apoptosis. An independent study reported that *NOTCH1* mutant tumors found in two HNSCC patient derived xenografts (PDXs) were sensitive to a PI3K inhibitor *in vivo* (Keysar et al., 2013). The preclinical data produced by our lab suggests that HNSCC patients with *NOTCH1* mutations may have an even better response to PI3K/mTOR inhibitors than those patients that harbor *PIK3CA* mutations.

1.8 Potential NOTCH1 and PI3K-PTEN-AKT/mTOR interactions

The interaction between the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways is well known in T-ALL. Constitutively active NOTCH1 signaling contributes to more than half of all cases of T-ALL (Weng et al., 2004). Initially, using γ -secretase inhibitors (GSIs) to block the proteolytic cleavage of NOTCH1 proved effective in inducing cellular growth arrest and apoptosis in some T-ALL cell lines. However, the GSI treatment failed to prevent the growth of *NOTCH1* mutant T-ALL cell lines. The findings suggest a mechanism of resistance that *NOTCH1* mutant T-ALL cell lines employ to overcome various therapies. Subsequent research demonstrates that in T-ALL cell lines, a key indicator of resistance to GSI-mediated NOTCH1 inhibition is homozygous loss of *PTEN*. Studies further show that *PTEN* expression is negatively regulated by HES1, a pivotal downstream target of the NOTCH1 pathway (Palomero et al., 2007). Additionally, γ -secretase-mediated NOTCH1 inhibition was shown to be synergistic with the inhibition of mTOR, a downstream effector of PI3K/AKT signaling, in T-ALL cell lines. The results were irrespective of the cell lines' sensitivity to the GSI treatment (Chan et al., 2007).

In some T-ALL cases, for instance, active NOTCH1 signaling leads to the transcriptional repression of PTEN and subsequent activation of the PI3K/AKT/mTOR pathway, which leads to cell survival and proliferation. In other T-ALL cases, NOTCH1 inhibition leads to upregulated PTEN that inhibits the PI3K/AKT/mTOR pathway and results in cell growth arrest and apoptosis. Additionally, T-ALL tumor cells survive and proliferate when both pathways are activated and no PTEN is present. Furthermore, T-ALL tumors that lack PTEN and active NOTCH1 signaling retains active PI3K/AKT/mTOR pathway signaling that promotes cellular survival and proliferation (Gutierrez and Look, 2007). The findings support a common indication: PTEN must be

present in the cell to inhibit the PI3K/AKT/mTOR pathway and prevent cell proliferation and survival.

Presumably, T-ALL tumors that possess mutations or deletions in PTEN are capable of constitutively inhibiting PTEN via the NOTCH1-HES1 pathway. The genetic alteration renders the cells resistant to GSIs by preventing the reactivation of PTEN, which typically occurs when NOTCH signaling is blocked. The findings also suggest that PTEN serves as a prominent mediator of communication between the NOTCH1 and PI3K/AKT/mTOR pathways in T-ALL. The interaction between both pathways is largely unknown in HNSCC. Because NOTCH1 has a different role in T-ALL than it does in HNSCC, the interactions may also be distinct. Elucidating the mechanism of communication between the NOTCH1 and PI3K/AKT/mTOR pathways in HNSCC may aid in the development of target therapies for patients with HNSCC tumors. After all, there are no effective targeted therapies for cancers driven by the loss of tumor suppressive properties.

1.9 Central Hypothesis and Specific Aims

The central goal of the project was to elucidate the mechanism of communication between the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways in HNSCC. More specifically, we investigated whether the NOTCH1 pathway regulates the activity of the PI3K-PTEN-AKT/mTOR pathway in HNSCC *in vitro*. Figure 5 is a diagram of the potential interaction between the NOTCH1 pathway and the PI3K-PTEN-AKT/mTOR pathway in HNSCC *in vitro*. We hypothesized that NOTCH1-mediated upregulation of HES1 promotes downregulation of PTEN transcription and activation of the PI3K/AKT/mTOR pathway in HNSCC.

Specific Aim 1: To determine if HES1 is regulated by NOTCH1 signaling in HNSCC cell lines.

A. To determine if basal levels of HES/HEY family members are different in *NOTCH1* mutant and wild-type HNSCC cell lines

We collaborated with the Department of Bioinformatics and Computational Biology who applied modified two-sample t-tests analyses to cleaved NOTCH1 (NICD) reverse phase protein array (RPPA) values and HES/HEY RNA sequence (RNA-seq) values to identify differentially expressed variables between the comparative groups. They also applied Spearman correlation and Pearson correlation analyses to the data sets to identify correlated variables between the comparative groups. *We hypothesized that there would be a direct positive correlation between mRNA expression of each of the HES and HEY family members and cleaved NOTCH1 status.*

B. To determine if levels of HES1 change when we inhibit NOTCH1 signaling in *NOTCH1* wild-type HNSCC cell lines

We employed three methods of manipulation to inhibit NOTCH1 signaling in *NOTCH1* wild-type HNSCC cell lines. We (1) blocked NOTCH1 signaling using OMP-52M51, a

NOTCH1 monoclonal blocking antibody; (2) silenced *NOTCH1* gene expression using siRNA targeting the *NOTCH1* receptor gene; and (3) obtained *NOTCH1*^{-/-} cell lines from Dr. Mitchell Frederick's laboratory. We measured NOTCH1 pathway, HES1, and PI3K-PTEN-AKT/mTOR pathway protein levels via western blot. We also measured *NOTCH1*, *HES1*, and *PTEN* mRNA levels via qRT-PCR. *We hypothesized that inhibiting NOTCH1 signaling would downregulate HES1 gene and protein expression, leading to upregulated PTEN transcription and protein activity, and the subsequent inactivation of the PI3K/AKT/mTOR pathway.*

C. To determine if levels of HES1 change when we induce NOTCH1 signaling in *NOTCH1* mutant HNSCC cell lines

We administered doxycycline to induce the NOTCH1 intracellular domain (NICD) expression system in *NOTCH1* mutant HNSCC cell lines. We measured NOTCH1 pathway, HES1, and PI3K-PTEN-AKT/mTOR pathway protein levels via western blot. We also measured *NOTCH1*, *HES1*, and *PTEN* mRNA levels via qRT-PCR. *We hypothesized that restoring active NOTCH1 signaling would upregulate HES1 gene and protein expression, leading to downregulated PTEN transcription and protein activity, and the subsequent hyper-activation of the PI3K/AKT/mTOR pathway.*

Specific Aim 2: To determine if HES1 regulates the PI3K-PTEN-/AKT/mTOR pathway.

A. To determine if PI3K-PTEN-AKT/mTOR pathway target protein levels change when we silence *HES1* gene expression in *NOTCH1* wild-type HNSCC cell lines
We silenced *HES1* gene expression in *NOTCH1* wild-type HNSCC cell lines using siRNA targeting the *HES1* gene. We measured NOTCH1 pathway, HES1, and PI3K-PTEN-AKT/mTOR pathway protein levels via western blot. We also measured *NOTCH1*, *HES1*, and *PTEN* mRNA levels via qRT-PCR. *We hypothesized that silencing the HES1 gene*

would downregulate HES1 protein expression, upregulate PTEN transcription and protein activity, and lead to the subsequent inactivation of the PI3K/AKT/mTOR pathway.

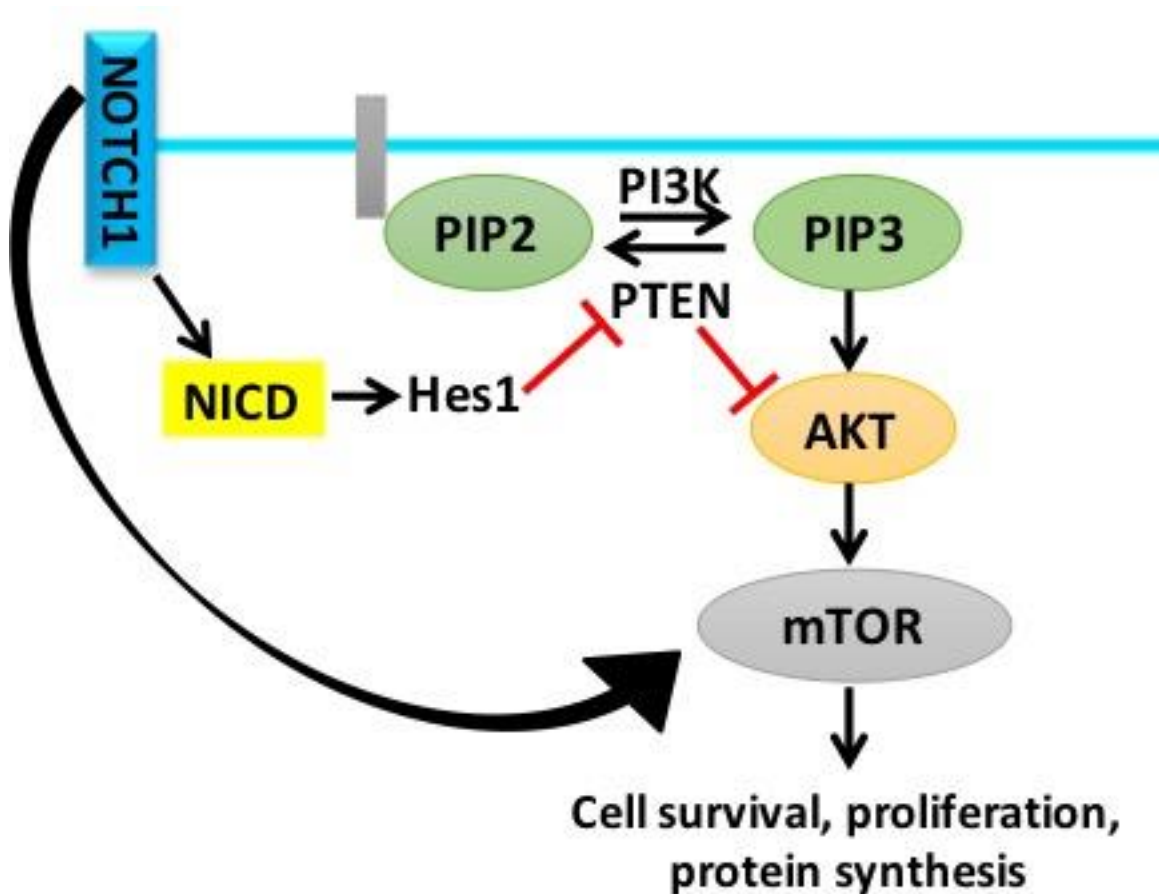


Figure 5: Potential NOTCH1 and PI3K-PTEN-AKT/mTOR pathways interaction.

Active NOTCH1 signaling will lead to the active transcription of *HES1* and subsequently upregulate *HES1* gene expression. In turn, *HES1* will transcriptionally repress *PTEN* transcription and subsequently downregulate *PTEN* gene expression. The ablated activity of PTEN will lead to the hyper-activation of the PI3K/AKT/mTOR pathway.

(Figure: Vaishnavi Sambandam, PhD).

CHAPTER 2: MATERIALS AND METHODS

2.1 HNSCC Cell Lines

A panel of 68 HNSCC cell lines were obtained from sources delineated in (Zhao et al., 2011) and maintained as directed. The cell lines were profiled for authenticity by Short Tandem Repeat (STR) analysis. They were tested for mycoplasma contamination using a Mycoplasma detection kit (Lonza). All cell lines were mycoplasma free at the time of testing. The assembly, characterization, and STR profiles for all of the cell lines are also described in (Zhao et al., 2011). The CRISPR/Cas9 knock out cell lines and the doxycycline inducible Notch1 intracellular domain (NICD) expression system were developed by Dr. Mitchell Frederick's laboratory.

2.2 Cell Culture

All cells were cultured in 1X Dulbecco's Modified Eagle's Medium (DMEM) complete media containing 10% FBS, 2% Penicillin/Streptomycin, L-glutamine, glucose, and sodium pyruvate (Corning). Cells were incubated in a 37°C, 5% carbon dioxide incubator.

2.3 Western blotting

HNSCC cell lines were lysed with 1X Cell Lysis Buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin; Cell Signaling Technology). After lysing, cells were incubated on ice and allowed to solubilize in the buffer for 15 minutes. The lysates were then centrifuged at 14,000 x g at 4°C for 15 minutes. The supernatant was collected and protein concentration was measured using the Pierce BCA protein assay kit (ThermoFisher Scientific). The lysates were then mixed with 10% β -mercaptoethanol and 4X Laemmli protein sample buffer (Bio-Rad) and loaded onto 4-

20% gradient gels (Mini-PROTEAN TGX Precast Gels; Bio-Rad), based on the molecular weights of the probed proteins. Gels were run using 10X premixed gel electrophoresis buffer (25mM Tris, 192 mM glycine and 0.1%SDS at pH 8.3, after mixing with distilled water; Bio-Rad) at 115 V for 1.5-2 hours. Gels were then turbo-transferred on nitrocellulose membranes using the Trans-Blot Turbo 5X Transfer Buffer (Bio-Rad) for 30 minutes in the Trans-Blot Turbo Transfer System (Bio-Rad). After transfer, the membranes were blocked using 5% milk in TBST for 2 hours with constant rotating agitation. The membranes were then incubated with the respective primary antibodies at various concentrations, ranging from 1:1000-1:10000, depending on the binding affinities of the antibodies. The membranes were then incubated overnight at 4°C with constant rotating agitation. After incubation, the membranes were washed for 15 minutes with washing buffer, TSBT containing 0.1% Tween-20, approximately 3 times. Membranes were then incubated with the respective species-specific horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature with constant rotating agitation. Protein signals were developed using the Pierce ECL Western Blotting Substrate or the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific), depending on the strength of the protein signals, on an X-ray film. Relative protein abundance was quantified using ImageJ (Schneider et al., 2012) and the expression of each protein was normalized against the internal control protein, β -actin, using the formula, $[(\text{target protein value})/(\text{actin value})]$. The fold change of protein expression was calculated using the formula, $[(B)/(A)]$, in which “B” represents the treated condition of each protein normalized against β -actin and “A” represents the control condition of each protein normalized against β -actin.

2.4 Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen). Cells were briefly scrapped from the petri dish using a scraper after adding the Buffer RLT Plus RNeasy Plus lysis buffer solution (Qiagen). The sample lysates were homogenized for 3 minutes using a QIAshredder (Qiagen). The lysates were then mixed with ethanol and applied to a silica-based filter, which selectively binds mRNA and larger rRNA. The filter was washed with Buffer RW1 and Buffer RPE wash buffers (Qiagen) to remove residual DNA and other contaminants. The RNA is then eluted from the filter with nuclease-free water. RNA concentration was measured using the NanoDrop 2000c spectrophotometer (Thermo Scientific). cDNA was then synthesized by reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad) in a MJ Mini personal thermal cycler (Bio-Rad) as directed by the manufacturer. A total of 100ng of cDNA was used per reaction, and each reaction was measured in triplets using a C1000 Touch Thermal Cycler (Bio-Rad). The expression of each target gene was normalized against the internal control gene, GAPDH, calculated using the formula, $2^{-(\text{GAPDH value}) - (\text{target protein value})}$. The fold change of gene expression was calculated using the formula, $[(B)/(A)]$, in which “B” represents the treated condition of each gene normalized against GAPDH and “A” represents the control condition of each gene normalized against GAPDH.

2.5 Transfection

siRNA transfections were performed using HNSCC cells. Cells were plated at a density of 2.0×10^6 the day before transfection. On the day of the transfection, Lipofectamine RNAiMAX transfection reagent (Invitrogen, Life Technologies) was diluted in 1X OPTI-MEM Reduced Serum Media (Gibco, Life Technologies). In a separate Eppendorf tube, the respective siRNA was diluted in 1X OPTI-MEM Reduced Serum

Media. The diluted siRNA solution was then added to the diluted Lipofectamine RNAiMAX reagent solution (1:1 ratio) and incubated at room temperature for 5 minutes. The siRNA-lipid complex was then added to the cells, which were incubated in a 37°C, 5% carbon dioxide incubator for 24 hours. Protein lysates were then collected at the 24-hour time point and analyzed via western blot and qRT-PCR.

2.6 Chemical inhibition

Brontictuzumab (OMP-52M51), a humanized IgG monoclonal antibody against NOTCH1, was obtained from OncoMed Pharmaceuticals, Inc. Normal mouse IgG, an isotype control immunoglobulin, was obtained from Santa Cruz Biotechnology, Inc (# sc-2025). Drug treatments via chemical inhibition were performed using HNSCC cells. Cells were plated at a density of 2.0×10^6 the day before drug administration. On the day of the treatment, OMP-52M51 and normal mouse IgG were added to the experimental and control cells, respectively, at a concentration of 0.5ug/ml. The cells were then incubated in a 37°C, 5% carbon dioxide incubator for 30 minutes, 2 hours, 4 hours, 8 hours, and 24 hours. Protein lysates were then collected at each of the time points and analyzed via western blot and qRT-PCR.

2.7 siRNAs for *NOTCH1* and *HES1*

The non-targeting control and *NOTCH1* siRNAs were obtained from Dharmacon (GE Life Sciences). Each pool contained four sequences for each gene. The antisense sequences for the ON-TARGETplus Non-targeting Pool are:

- 1) UGGUUUACAUGUCGACUAA
- 2) UGGUUUACAUGUUGUGUGA
- 3) UGGUUUACAUGUUUUCUGA

4) UGGUUUACAUGUUUUUCCUA

The antisense sequences for the NOTCH1 ON-TARGETplus SMARTpool are:

1) GCGACAAGGUGUUGACGUU

2) GAUGCGAGAUCGACGUCAA

3) GGACAUCACGGAUCAUAUG

4) GAACGGGGCUAACAAAGAU

The *HES1* siRNA was obtained from Santa Cruz Biotechnology, Inc (# sc-270146). The construct consists of a pool of three target-specific, 19-25 nucleotide sequences designed to silence gene expression.

2.8 Antibodies

The antibodies utilized in this project are outlined in Table 1.

Antibody	Source	Catalog No.	Conc.	Diluent
NOTCH1-NTM	Cell Signaling	3439	1:1000	5% BSA + 0.03% NaN ₃ – TBST
NOTCH2-NTM	Cell Signaling	5732	1:1000	5% BSA + 0.03% NaN ₃ – TBST
NOTCH3-NTM	Cell Signaling	5276	1:1000	5% BSA + 0.03% NaN ₃ – TBST
NOTCH4-NTM	Cell Signaling	2423	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Cleaved NOTCH1	Cell Signaling	4147	1:1000	5% BSA + 0.03% NaN ₃ – TBST
HES1	Cell Signaling	11988	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Phospho-PTEN (Ser380/Thr382/383)	Cell Signaling	9549	1:1000	5% BSA + 0.03% NaN ₃ – TBST
PTEN	Cell Signaling	9552	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Phospho-AKT (Ser473)	Cell Signaling	4060	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Total AKT	Cell Signaling	4691	1:1000	5% BSA + 0.03% NaN ₃ – TBST

Phospho-S6 (Ser235/236)	Cell Signaling	2211	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Phospho-S6 (Ser240/244)	Cell Signaling	2215	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Total S6	Cell Signaling	2217	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Phospho-4EBP1 (Ser65)	Cell Signaling	13443	1:1000	5% BSA + 0.03% NaN ₃ – TBST
Total 4EBP1	Cell Signaling	9452	1:1000	5% BSA + 0.03% NaN ₃ – TBST
β-actin	Cell Signaling	3700	1:10000	5% BSA + 0.03% NaN ₃ – TBST

Table 1: Description of the antibodies used in the project.

2.9 Primers

The primers utilized in this project are outlined in Table 2.

Gene	Source	Target Sequence(5'-3')
GAPDH	Sigma-Aldrich	Forward: CCCATCACCATCTTCCAG Reverse: ATGACCTTGCCCACAGCC
NOTCH1	Sigma-Aldrich	Forward: TCCACCAGTTTGAATGGTCA Reverse: AGCTCATCATCTGGGACAGG
HES1	Sigma-Aldrich	Forward: CCCAACGCAGTGTCACCTTC Reverse: TACAAAGGCGCAATCCAATATG
PTEN	Sigma-Aldrich	Forward: CCAGGACCAGAGGAAACCT Reverse: GCTAGCCTCTGGATTGA

Table 2: Description of the primers used in the project.

CHAPTER 3: RESULTS AND ANALYSES

3.1 *HES5* mRNA expression is lower in *NOTCH1* mutant than *NOTCH1* wild-type HNSCC cell lines

Previous research suggests that HES1 transcriptionally represses *PTEN*, leading to the hyperactivity of the PI3K/AKT/mTOR pathway and increased cell growth, survival, and proliferation (Gutierrez and Look, 2007; Palomero et al., 2007). Because HES1 is a member of a family of transcriptional repressors (HES1-7; HEY1, HEY2, HEYL) that can be regulated by NOTCH, we investigated whether there was a significant correlation between cleaved NOTCH1 (NICD) status and the mRNA expression of the HES and HEY family members in HNSCC cell lines. All data was processed and analyzed by Dr. Jing Wang's team in the Department of Bioinformatics at M.D. Anderson Cancer Center. Statisticians applied modified two-samples t-tests to the data sets to identify differentially expressed variables between the comparative groups. They also applied Spearman correlation and Pearson correlation analyses to the data sets to identify correlated variables between the comparative groups.

We hypothesized that there would be a direct positive correlation between the mRNA expression of each of the HES and HEY family members and the cleaved NOTCH1 protein levels in the HNSCC cell lines. The scatterplot and barplot in Figure 6 shows that HES5 is the only member of the HES and HEY family of transcriptional repressors to exhibit increased mRNA levels in HNSCC cell lines possessing high cleaved NOTCH1 protein [spearman.pval: 0.007; adj.p.val: 0.067]. The scatterplots in Figure 7 further confirm the findings.

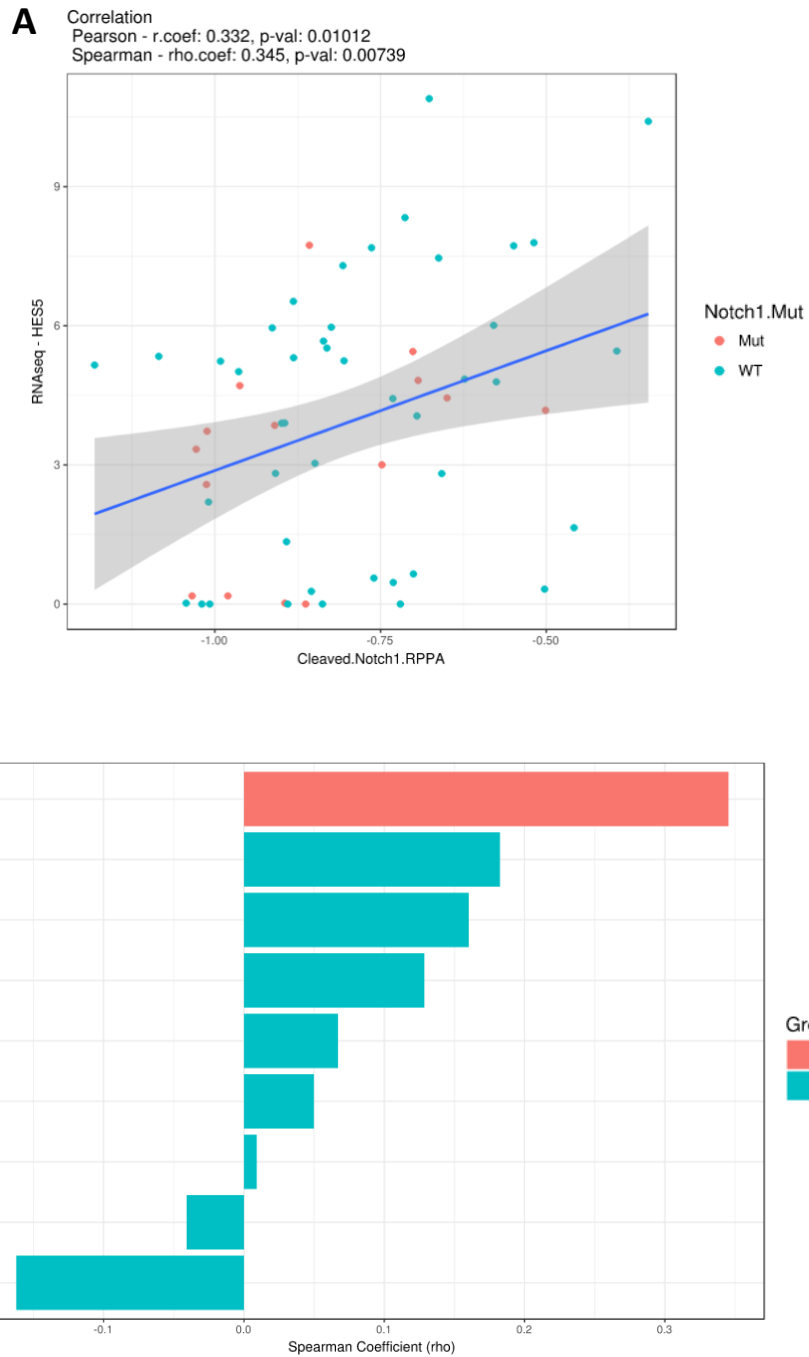


Figure 6: *HES5* mRNA expression significantly correlated with cleaved NOTCH1 (NICD) protein levels in HNSCC.

[A] Scatterplot comparing NOTCH1 activation status and HES/HEY mRNA expression.
 [B] Barplot comparing NOTCH1 activation status and HES/HEY mRNA expression.
 Measures of significance are color-coded, with coral bars constituting significant values and teal bars indicating insignificant values.

(Analysis: Li Shen, PhD & Jing Wang, PhD, Department of Bioinformatics and Computational Biology)

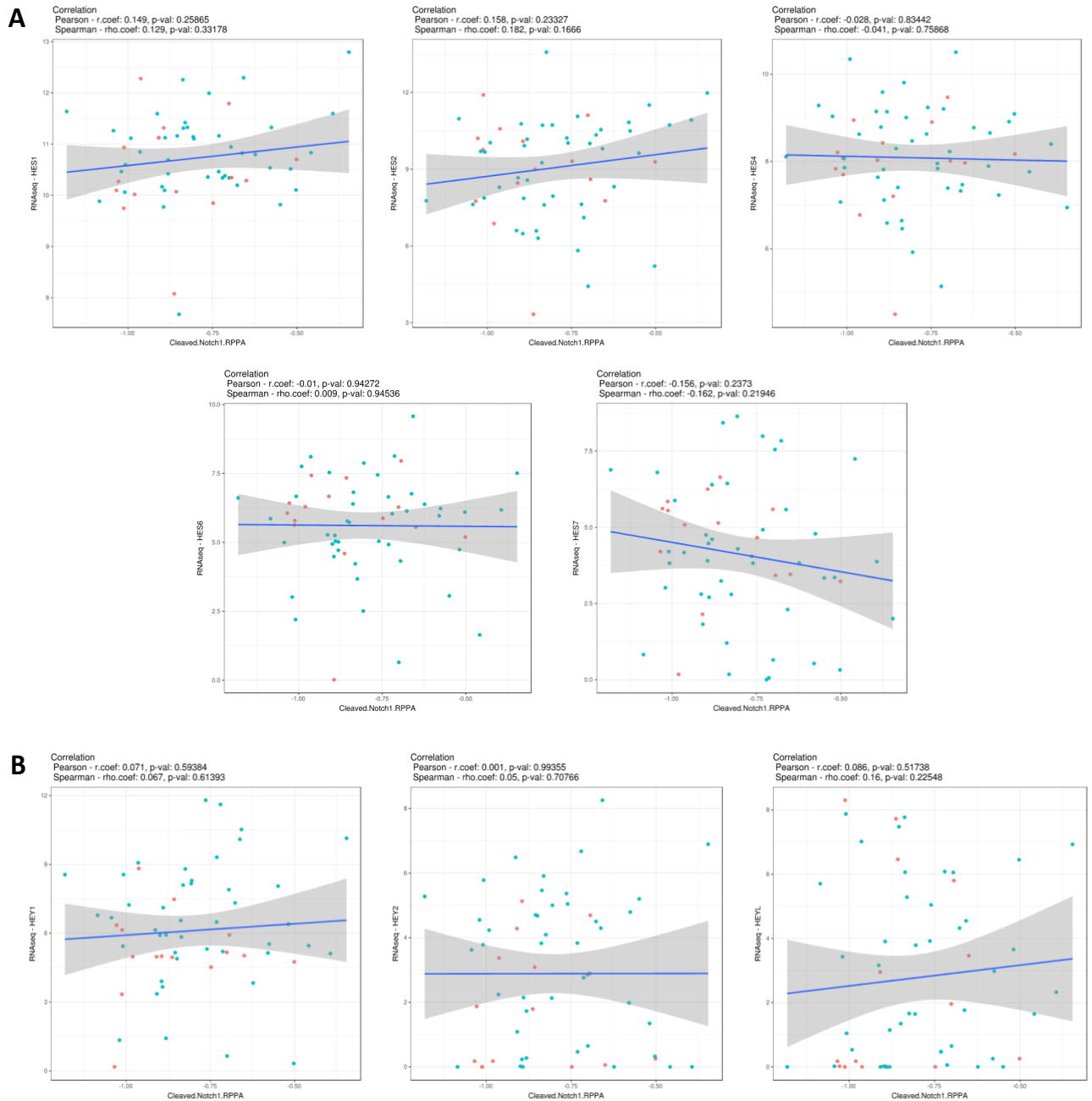


Figure 7: No significant correlation between HES/HEY mRNA expression and NOTCH1 activation status in HNSCC.

[A] Scatterplots comparing HES family mRNA expression, with the exception of HES5, and cleaved NOTCH1 (NICD) protein levels in HNSCC cell lines. [B] Scatterplots comparing HEY family mRNA expression and cleaved NOTCH1 (NICD) protein levels in HNSCC cell lines. *NOTCH1* mutant and *NOTCH1* wild-type HNSCC cell lines are indicated with coral and teal dots, respectively. (Analysis: Li Shen, PhD & Jing Wang, PhD, Department of Bioinformatics and Computational Biology)

3.2 *NOTCH1* wild-type HNSCC cell lines have diverse levels of NOTCH1 activation

HES1 is a well-established transcriptional target gene of the NOTCH1 pathway. In other words, active NOTCH1 signaling promotes the active transcription of HES1, leading to upregulated HES1 mRNA levels. Thus, HES1 mRNA levels were expected to be positively correlated with cleaved NOTCH1 protein levels in HNSCC cell lines. Because the results of the Spearman and Pearson correlation analyses were rather different from our hypothesis, we decided to examine the NOTCH1 activation of the *NOTCH1* wild-type HNSCC cell lines. We collected lysate from 3 *NOTCH1* wild-type HNSCC cell lines (FADU, PJ34, OSC19) after 24 hours and measured the basal protein levels of NOTCH1, NICD, HES1, and PTEN via western blot analysis.

We expected that *NOTCH1* wild-type HNSCC cell lines would display higher levels of NOTCH1 and NICD basal protein, suggesting increased NOTCH1 activation due to active NOTCH1 signaling, compared to mutant cell lines. Figure 8 reveals that the levels of cleaved NOTCH1 (NICD) protein vary across the *NOTCH1* wild-type HNSCC cell lines (with overlapping levels observed in mutants), indicating that the *NOTCH1* wild-type HNSCC cell lines have diverse NOTCH1 activation. The diverse NOTCH1 and NICD protein levels also yielded diverse HES1 and PTEN protein levels across the cell lines. For instance, while higher protein levels of NOTCH1 and NICD correlated with higher protein levels of HES1 in some cell lines, lower protein levels of NOTCH1 and NICD correlated with lower protein levels of HES1 in other cell lines. These results may explain why the mRNA levels of *HES1* did not correlate significantly with the protein levels of cleaved NOTCH1.

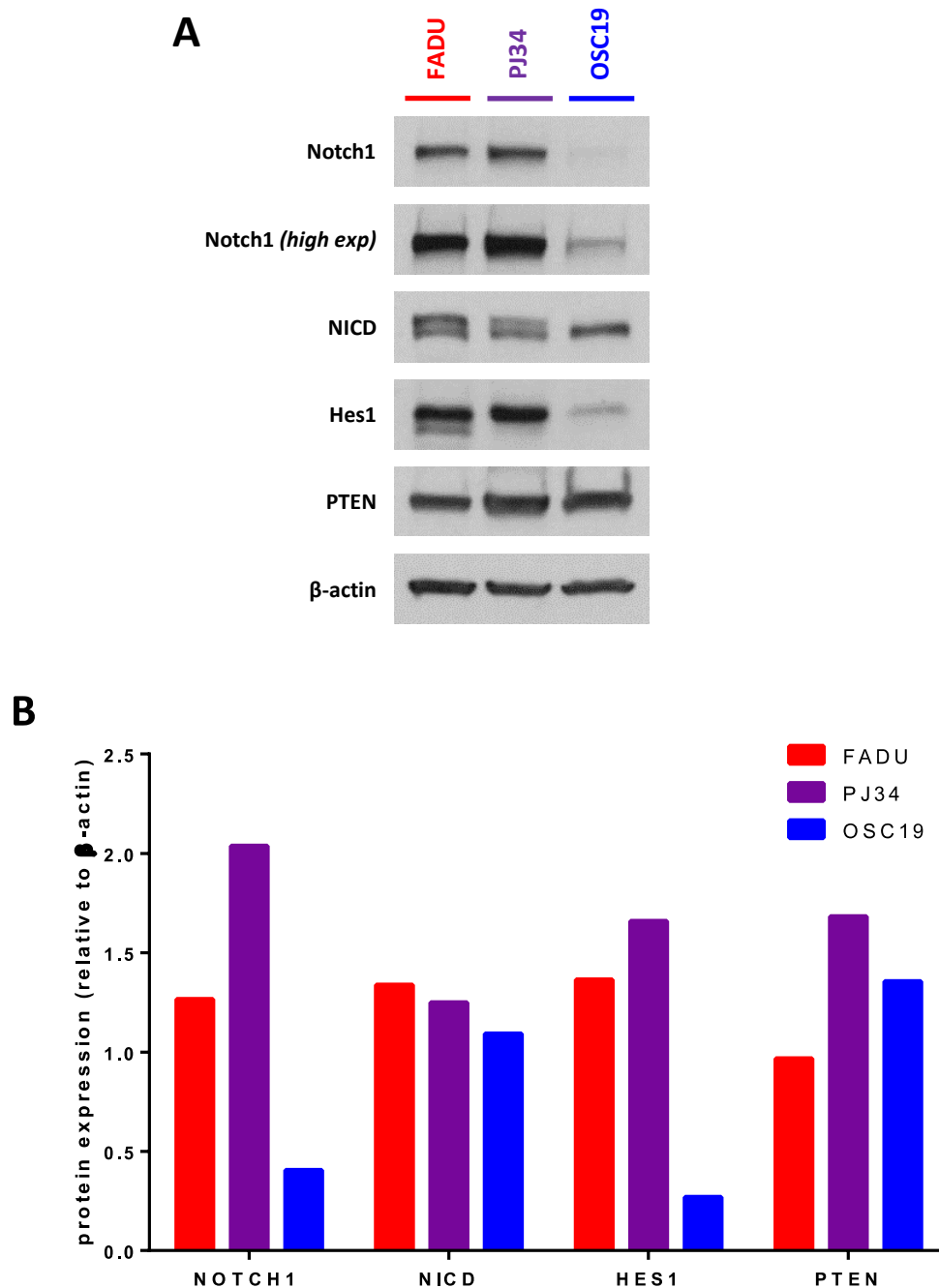


Figure 8: Diverse NOTCH1 activation in *NOTCH1* wild-type HNSCC cell lines.

[A] Western blot analysis of NOTCH1, NICD, HES1, and PTEN basal protein levels after 24 hours in *NOTCH1* wild-type HNSCC cell lines. [B] Bar graph comparing quantification of protein levels measured via western blot. *NOTCH1* wild-type HNSCC cell lines are color-coded. FADU, PJ34, and OSC19 are indicated in red, purple, and blue, respectively.

3.3 Inhibition of NOTCH1 signaling does not significantly affect the PI3K-PTEN-AKT/mTOR pathway in HNSCC

Preliminary experiments in our laboratory show that *NOTCH1* mutant HNSCC cell lines are sensitive to dual PI3K/mTOR inhibitors. To investigate the mechanism of sensitivity in the cell lines, we explored the interaction between the NOTCH1 pathway and the PI3K-PTEN-AKT/mTOR pathway. We treated 3 *NOTCH1* wild-type HNSCC cell lines (FADU, PJ34, and OSC19) at various time points (30 minutes, 2 hours, 4 hours, 8 hours, and 24 hours) with OMP-52M51 at a concentration of 0.5ug/ml. We treated the control groups of the same cell lines with normal mouse IgG, an isotype control immunoglobulin, using the same concentration at the same time points. We then collected lysate and measured NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels via western blot analysis. Experiments were repeated twice in each cell line for validity and reliability measures (n=2).

OMP-52M51, also known as Brontictuzumab, is a first-in-class humanized IgG monoclonal antibody against NOTCH1. The antibody exerts its function by binding to the negative regulatory region (NRR) of the extracellular domain on the NOTCH1 receptor and changing its conformation. The conformational change prevents the receptor from binding to an adjacent ligand and ultimately blocks NOTCH1 signaling (Patnaik et al., 2014). Figure 9 is a schematic of the functional activity of OMP-52M51 *in vitro* and *in vivo*. Although gamma secretase inhibitors are commonly used to inhibit NOTCH1 signaling, we wanted to use a more specific inhibitor to prevent NICD cleavage. Using the active signaling of NOTCH1 in T-ALL as a model, we postulated that inhibiting NOTCH1 signaling in *NOTCH1* wild-type HNSCC cell lines would lead to downregulated HES1 transcription and upregulated PTEN activity, thereby inactivating the PI3K/AKT/mTOR pathway. However, results of the experiment yielded a different

outcome. Figure 10 shows that inhibiting the NOTCH1 pathway has no significant effect on HES1, PTEN, or the PI3K/AKT/mTOR pathway. While NOTCH1 signaling was blocked effectively in each cell line, there were no significant or consistent differences among the respective protein levels of the control and treated samples across the various time points.

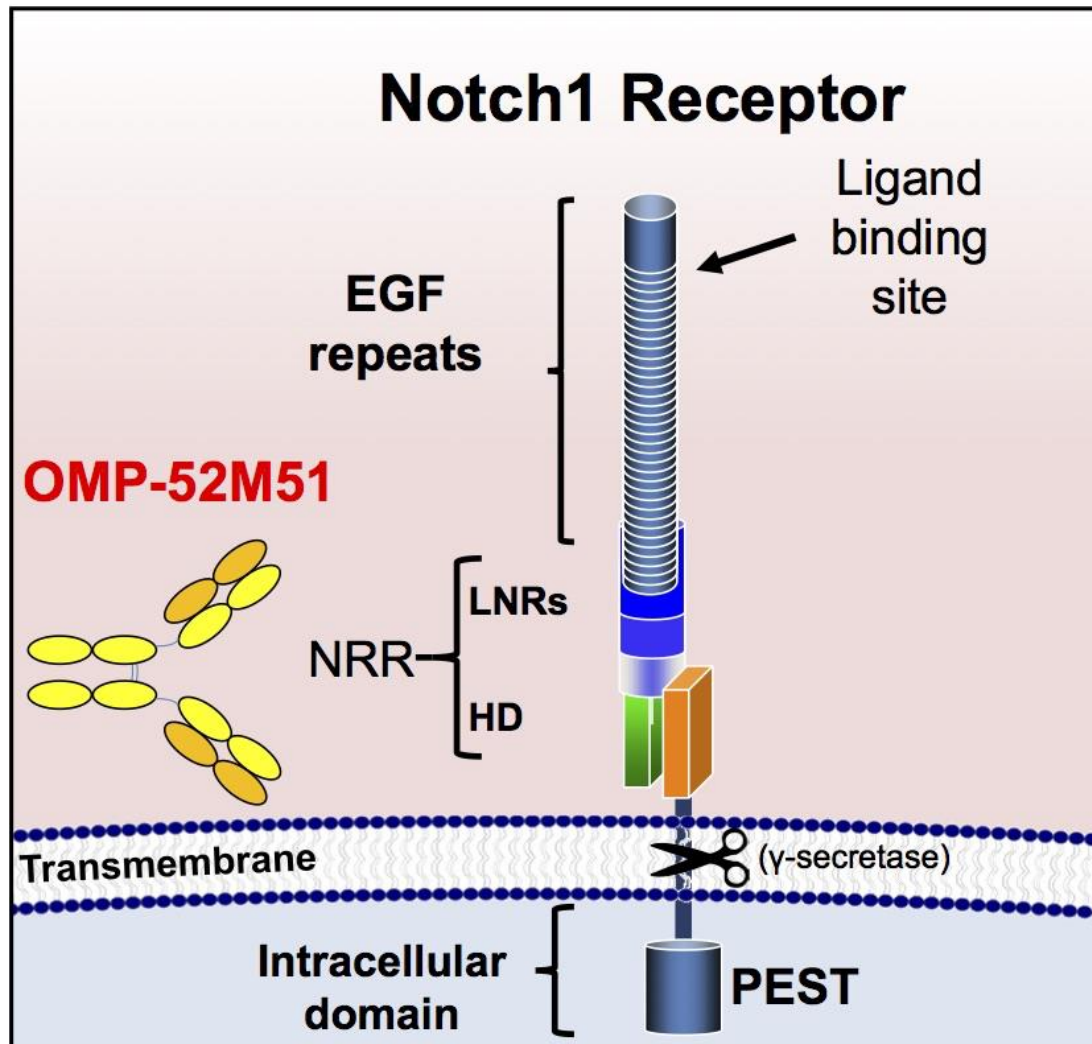
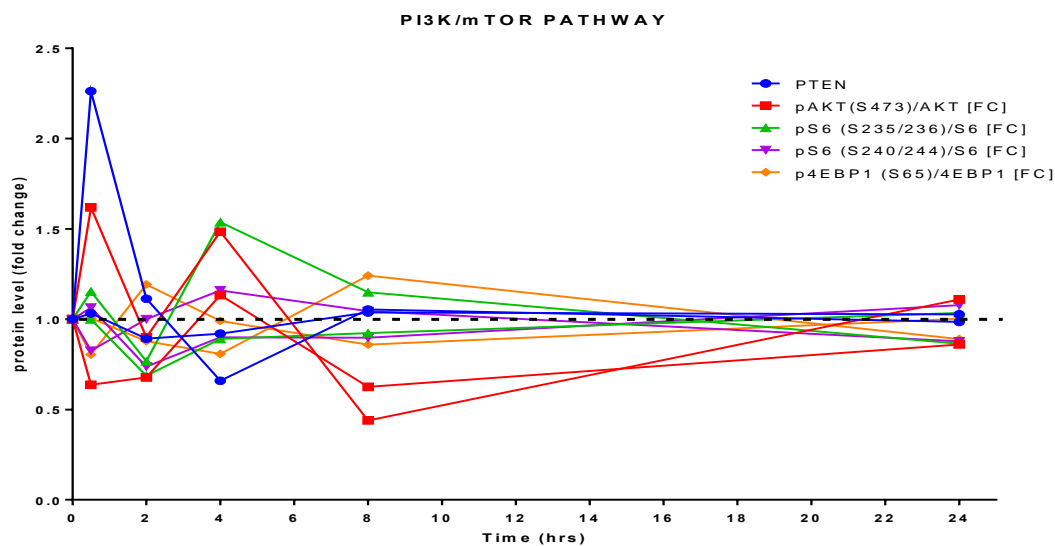
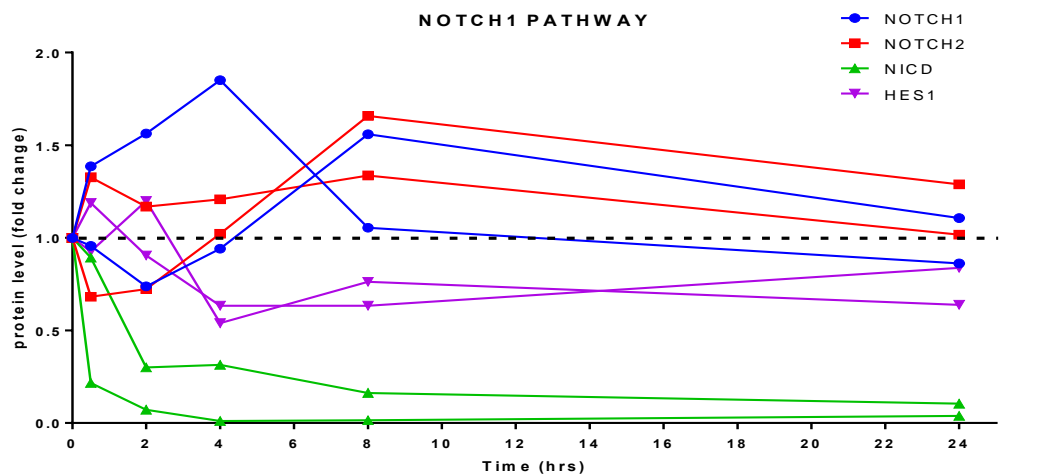
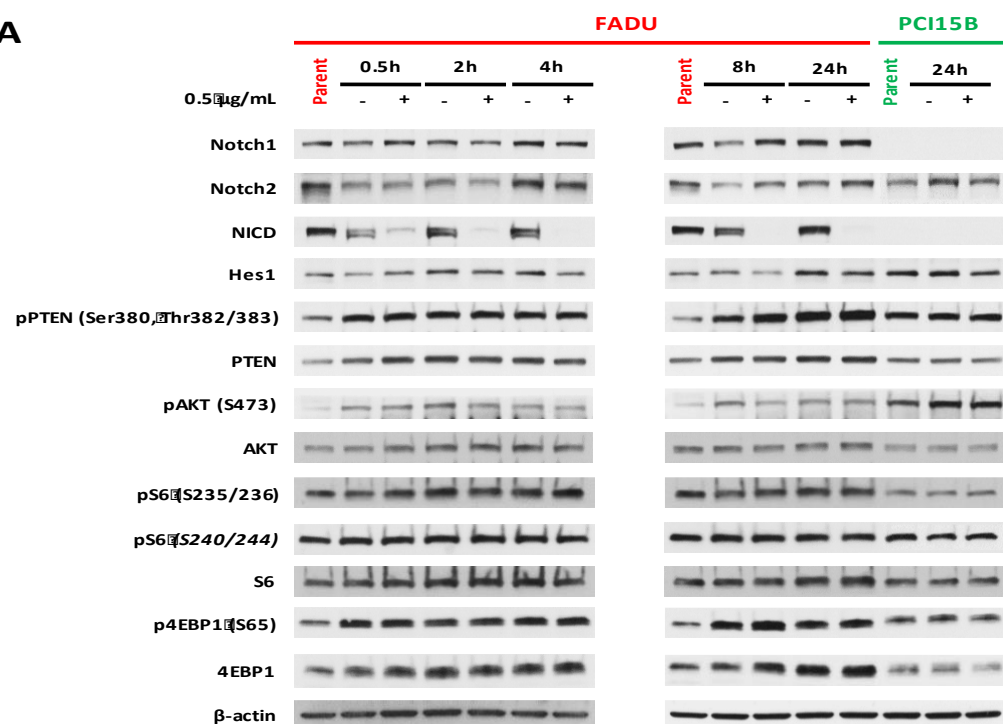


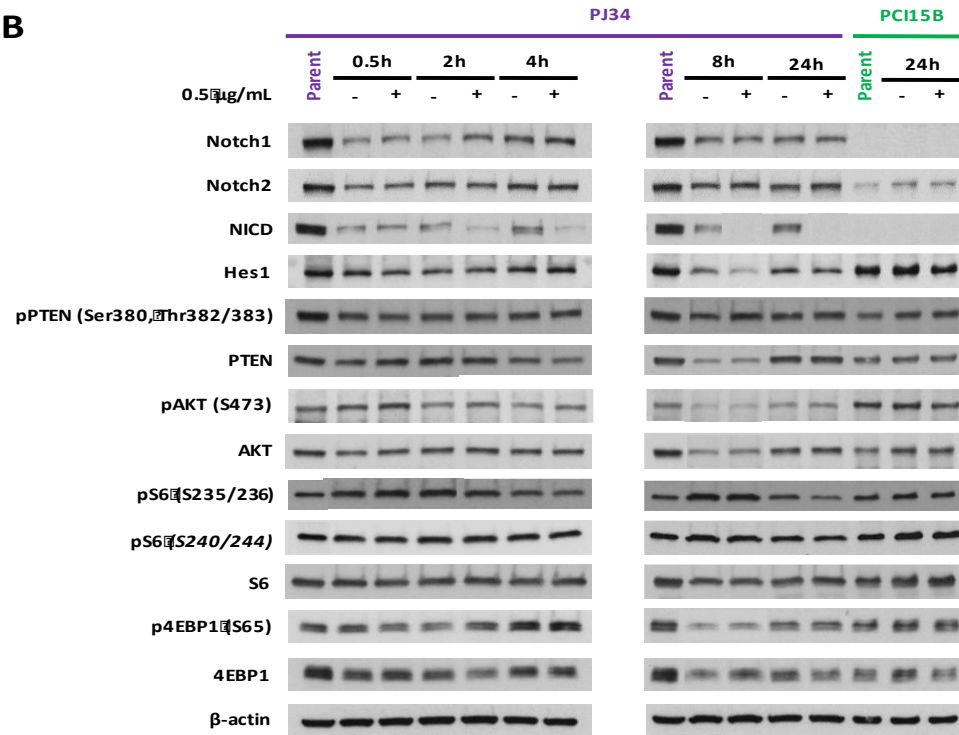
Figure 9: Activity of OMP-52M51 *in vitro* and *in vivo*.

Brontictuzumab, also known as OMP-52M51, is a first-in-class humanized IgG monoclonal antibody against NOTCH1. It binds to the negative regulatory region (NRR) of the NOTCH1 extracellular domain on the receptor and changes its conformation, preventing the receptor from binding to an adjacent ligand and blocking NOTCH1 signaling. *Permission obtained from Patnaik, A., LoRusso, P., Munster, P., Tolcher, A.W., Davis, S.L., Heymach, J., Ferrarotto, R., Xu, L., Kapoun, A.M., Faoro, L., Lewicki, J.A., Dupont, J., Eckhardt, S.G. (2014). EORTC-NCI-AACR Symposium. (Patnaik et al., 2014).*

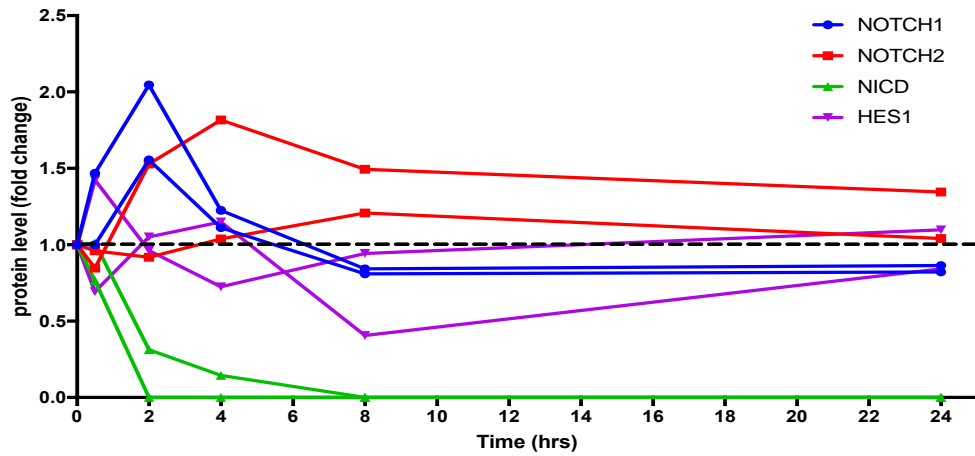
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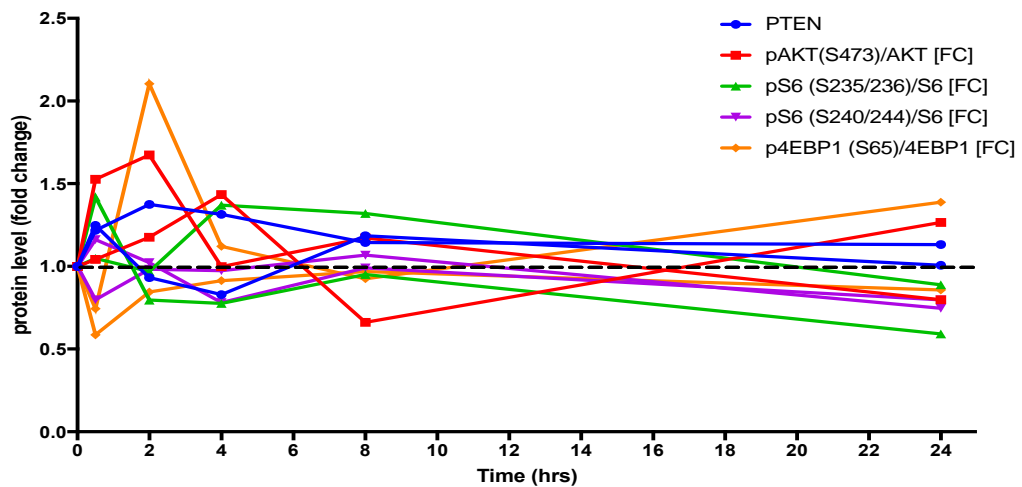
B



NOTCH1 PATHWAY



PI3K/mTOR PATHWAY



C

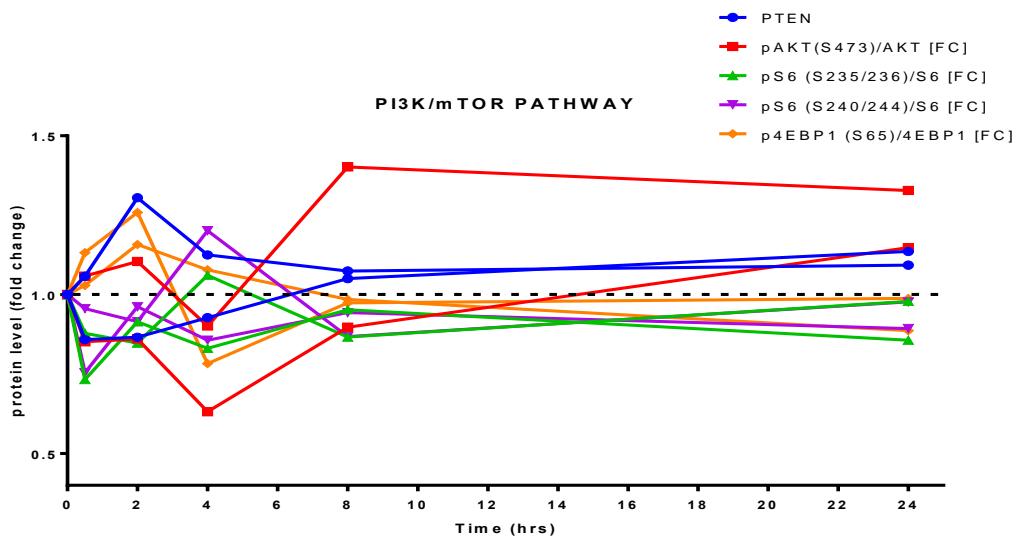
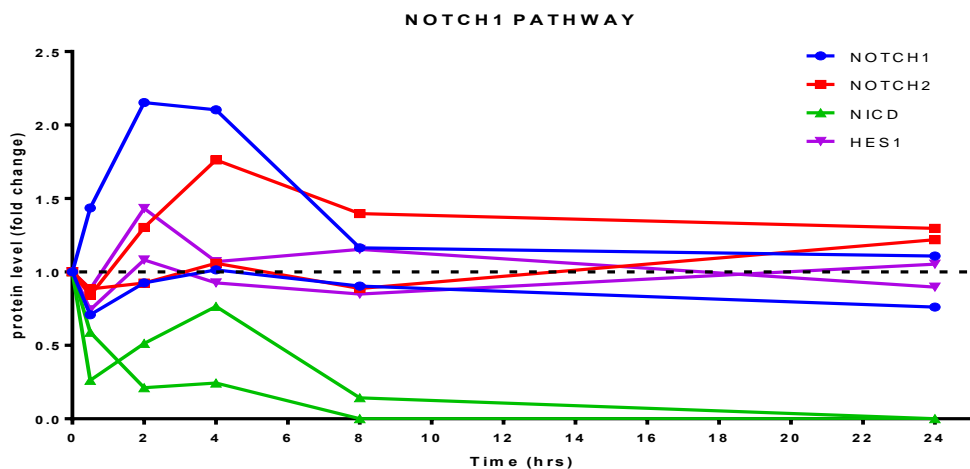
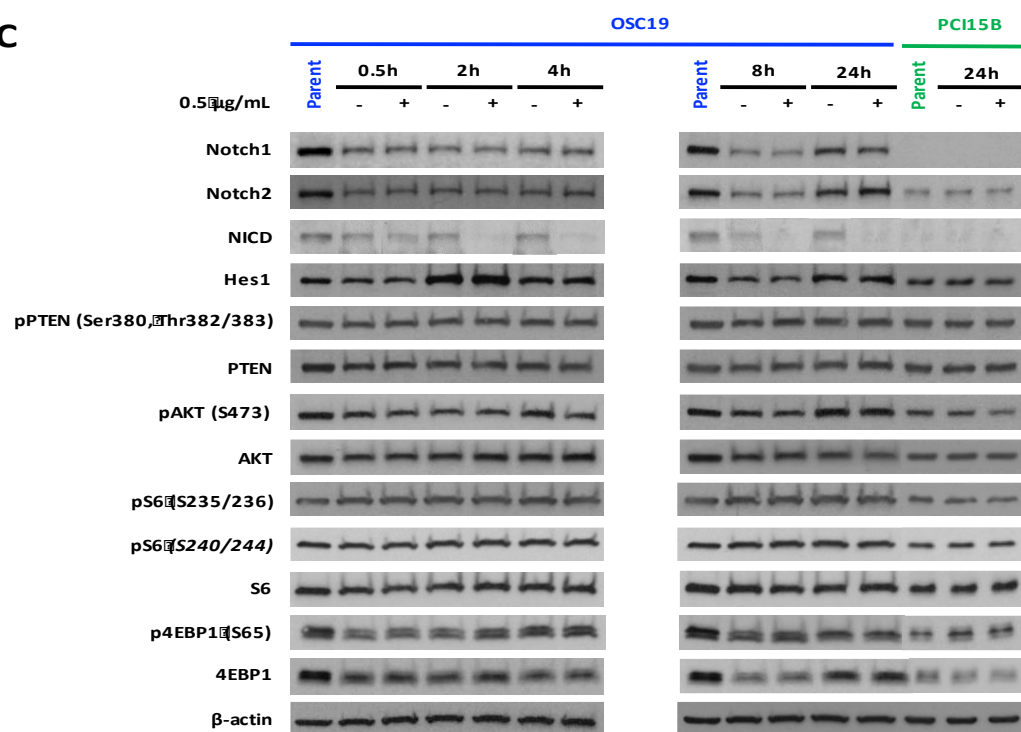


Figure 10: Blocking NOTCH1 signaling has no significant effect on the PI3K-PTEN-AKT/mTOR pathway.

Western blot analysis and quantification of NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels after 0.5ug/ml OMP-52M51 treatment for 30 minutes, 2 hours, 4 hours, 8 hours, and 24 hours in [A] FADU, [B] PJ34, and [C] OSC19. Pathway proteins are color-coded in line graphs.

3.4 Inhibition of NOTCH1 signaling has differential effects on *HES1* and *PTEN* transcription in HNSCC

Previous studies show that inhibiting NOTCH1 signaling in *NOTCH1* wild-type HNSCC cell lines has no significant or consistent effect on the PI3K-PTEN-AKT/mTOR pathway at the protein level. We investigated whether the same trend persisted at the mRNA level. Because results of the western blot analysis revealed that HES1 protein levels decreased the most after 8 hours of successful OMP-52M51 treatment, we repeated the treatment of FADU, PJ34, and OSC19 with 0.5ug/ml of OMP-52M51 for 8 hours and isolated RNA. We then measured *NOTCH1*, *HES1*, and *PTEN* mRNA levels via qRT-PCR. Experiments were repeated twice in each cell line for validity and reliability measures (n=2).

We hypothesized that inhibiting NOTCH1 signaling would downregulate *HES1* transcription, leading to decreased *HES1* mRNA levels and increased *PTEN* mRNA levels. Nevertheless, we discovered that the results varied among the cell lines (Figure 11). While *HES1* mRNA levels decreased in FADU and PJ34 after blocking NOTCH1 signaling, there was no significant effect on *HES1* mRNA levels in OSC19 after treatment. Of note, there is a subtle decrease in HES1 protein in FADU and PJ34, as seen in Figure 10, at the same timepoint in which we observe decreased *HES1* mRNA expression in the same cell lines. Moreover, blocking NOTCH1 signaling had no significant effect on *PTEN* transcription in FADU, PJ34, or OSC19. Taken together, the findings of the western blot and qRT-PCR analyses suggest that the well-established association between NOTCH1, HES1, and PTEN in T-ALL may be weak or nonexistent in HNSCC.

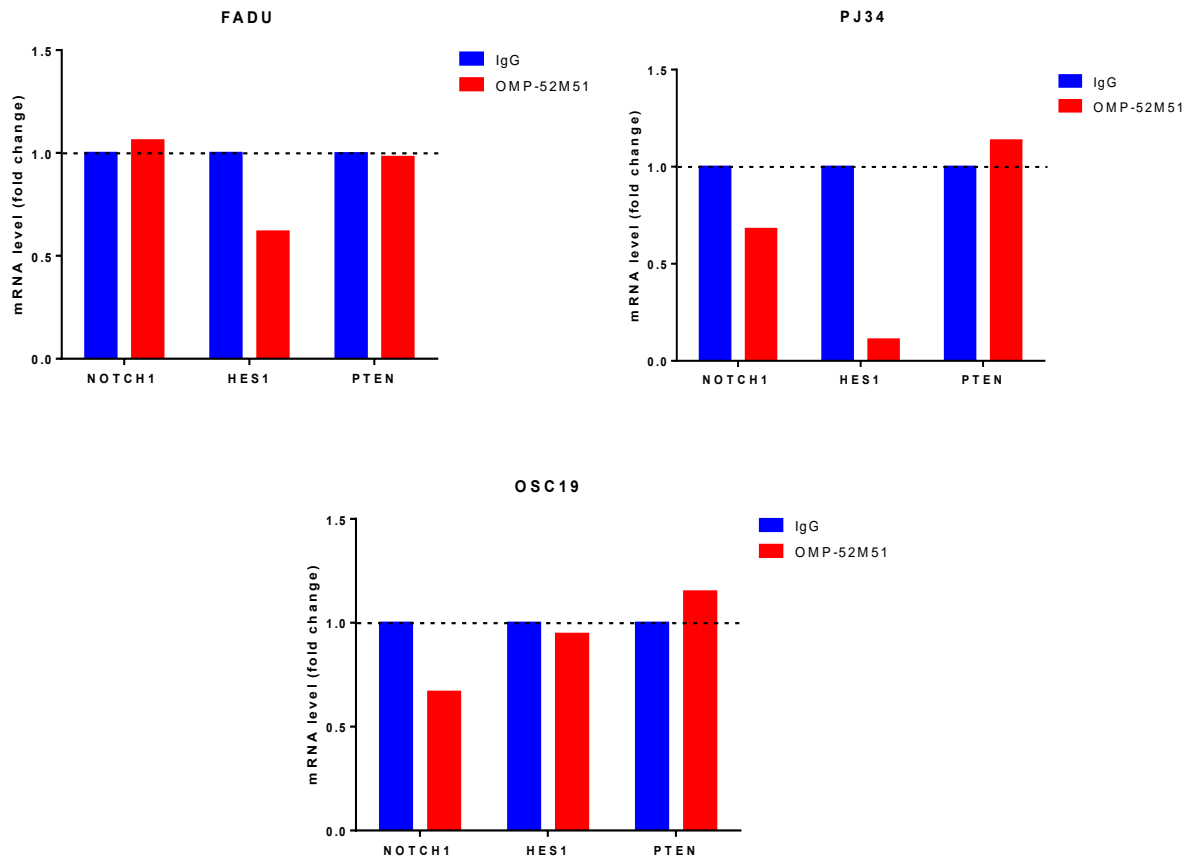


Figure 11: Blocking NOTCH1 signaling has differential effects on *HES1* and *PTEN* transcription in HNSCC.

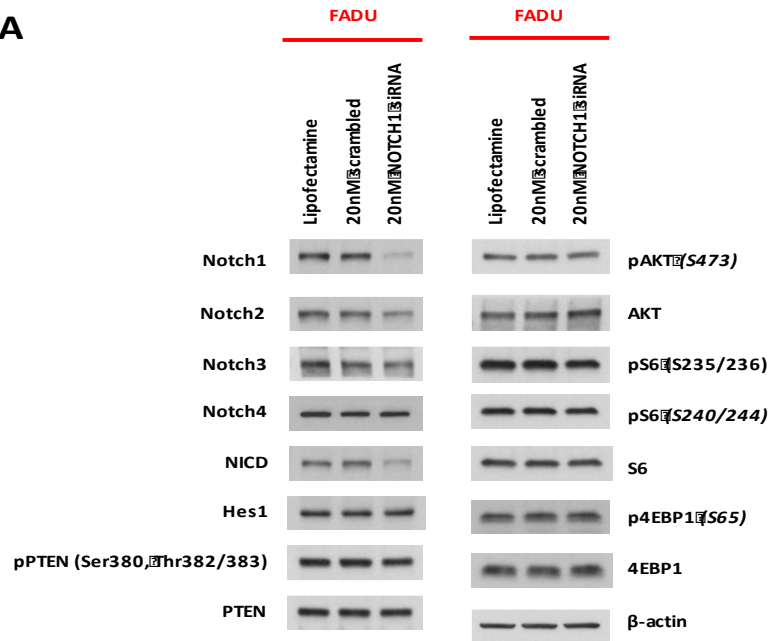
Quantification of *NOTCH1*, *HES1*, and *PTEN* mRNA levels after qRT-PCR analysis of FADU, PJ34, and OSC19. Cell lines were treated with OMP-52M51 at a concentration of 0.5ug/ml for 8 hours. Control and treated samples are denoted in blue and red, respectively.

3.5 Knock down (KD) of *NOTCH1* has no significant effect on the PI3K-PTEN-AKT/mTOR pathway in HNSCC

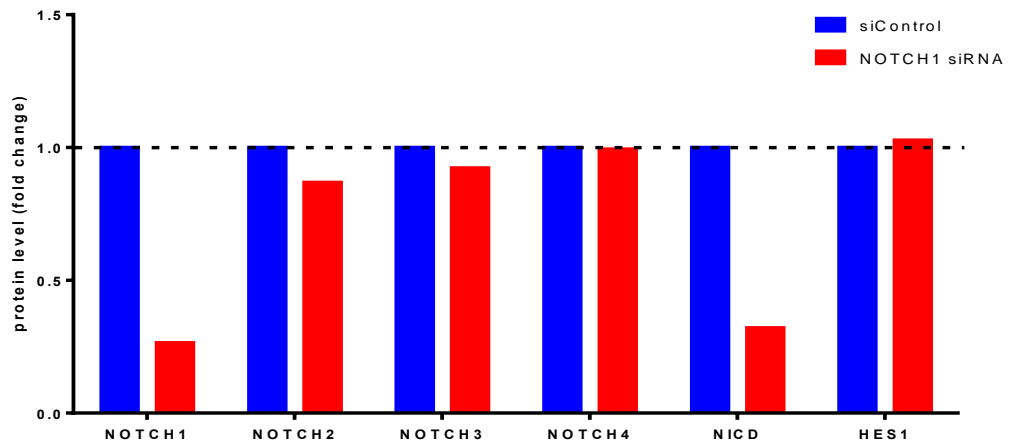
To test our hypothesis using an independent method, we decided to employ a more genetic means of inhibiting NOTCH1 signaling. We treated the 3 *NOTCH1* wild-type HNSCC cell lines (FADU, PJ34, and OSC19) with 20nM siRNA targeting the *NOTCH1* gene for 24 hours. NOTCH1 protein and mRNA have a half-life of approximately 1.5 hours, making 24 hours a suitable time point for silencing the *NOTCH1* gene (Nedjic and Aifantis, 2010). The control groups of the respective cell lines were treated with 20nM non-targeting siRNA for 24 hours. We then collected lysate and measured NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels via western blot analysis. Experiments were repeated twice in each cell line for validity and reliability measures (n=2).

We hypothesized that using siRNA to inhibit NOTCH1 signaling would lead to downregulated HES1 transcription and upregulated PTEN activity, thereby inactivating the PI3K/AKT/mTOR pathway in NOTCH1 wild-type HNSCC cell lines. However, Figure 12 shows that genetically blocking NOTCH1 signaling via siRNA has no significant effect on HES1, PTEN, or the PI3K/AKT/mTOR pathway. Although we successfully sustained approximately 70% knock down of the *NOTCH1* gene, there were no differences among the NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway proteins in the control and treated samples of each cell line.

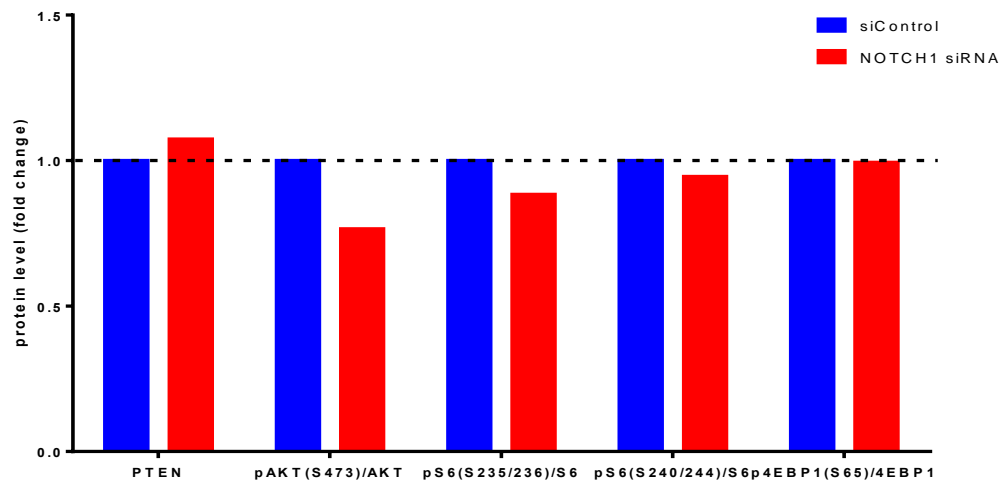
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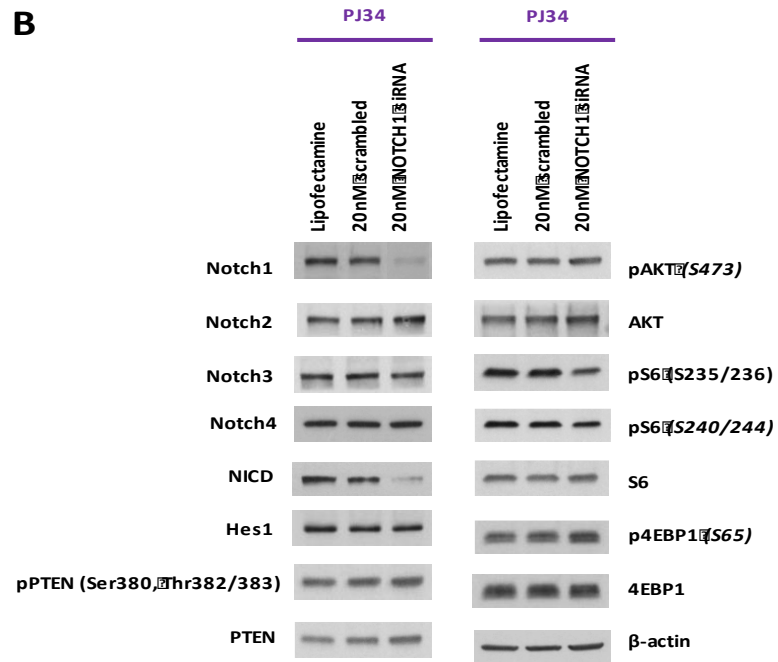
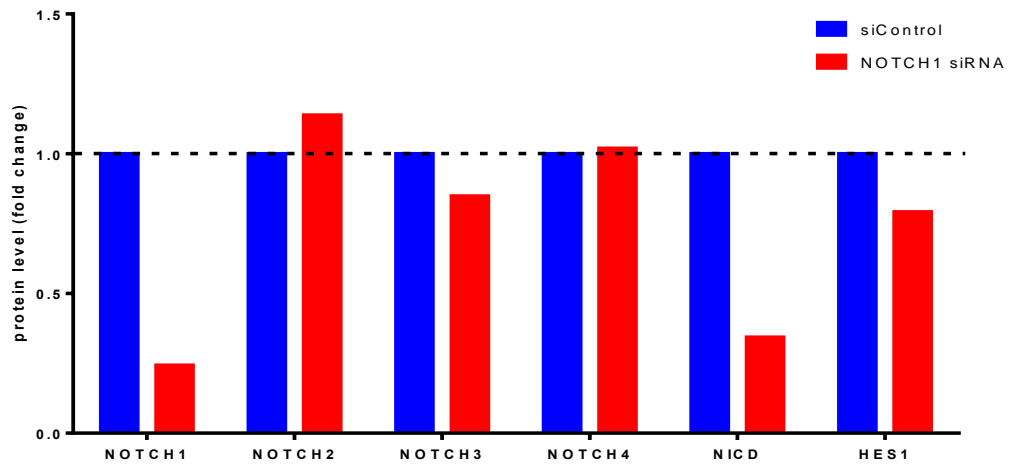
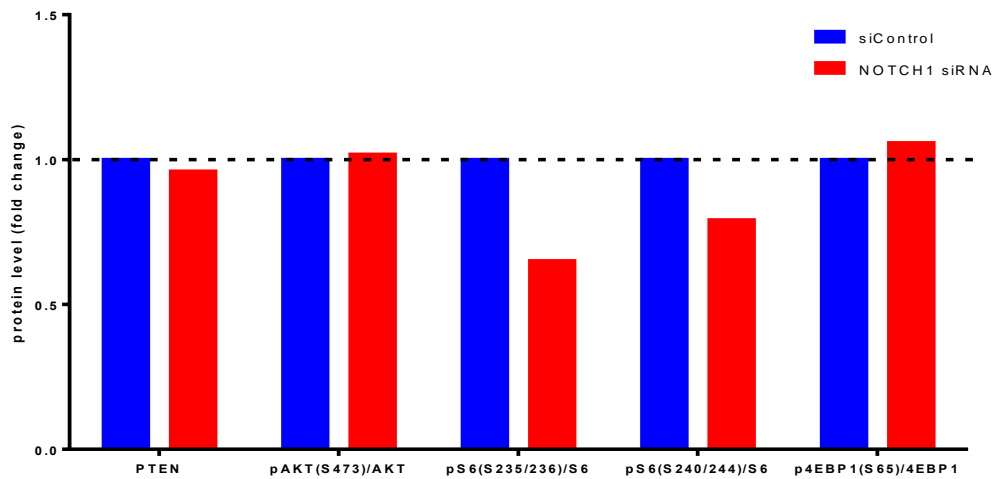


NOTCH1 PATHWAY

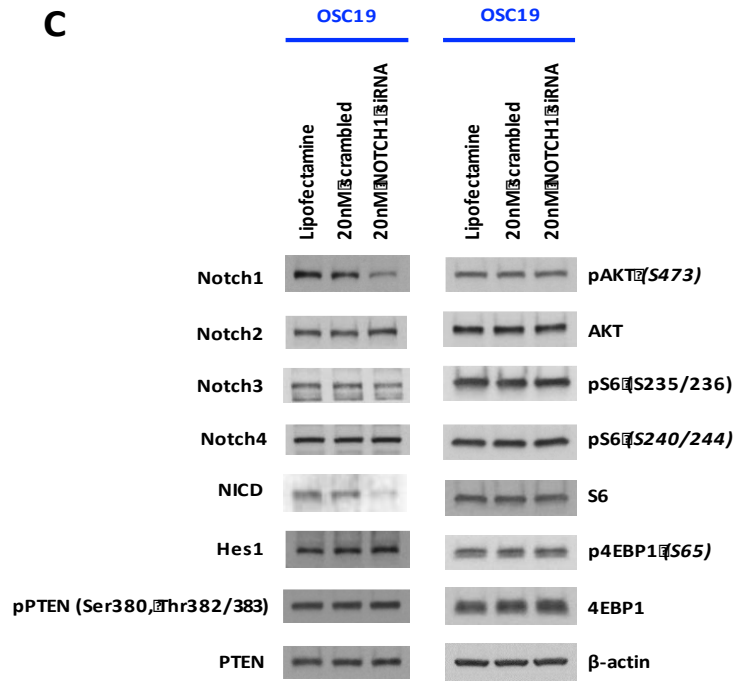


PI3K/mTOR PATHWAY

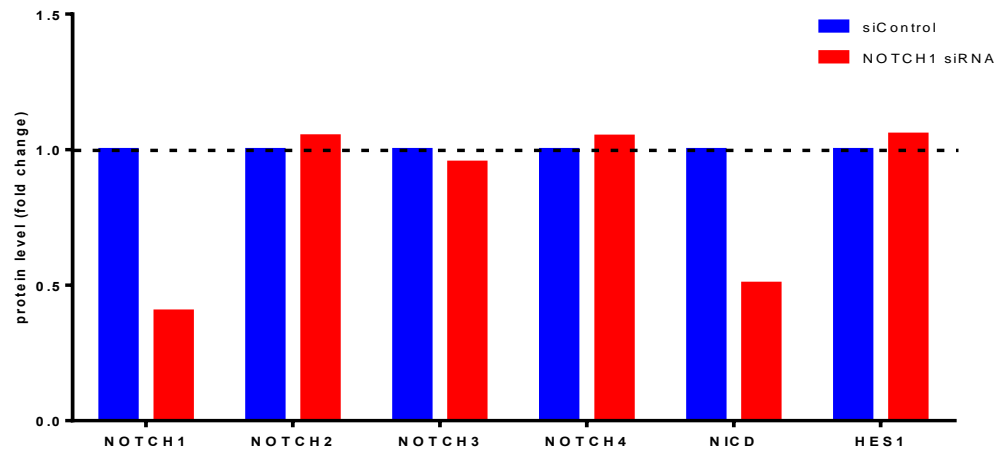


B**NOTCH1 PATHWAY****PI3K/mTOR PATHWAY**

C



NOTCH1 PATHWAY



PI3K/mTOR PATHWAY

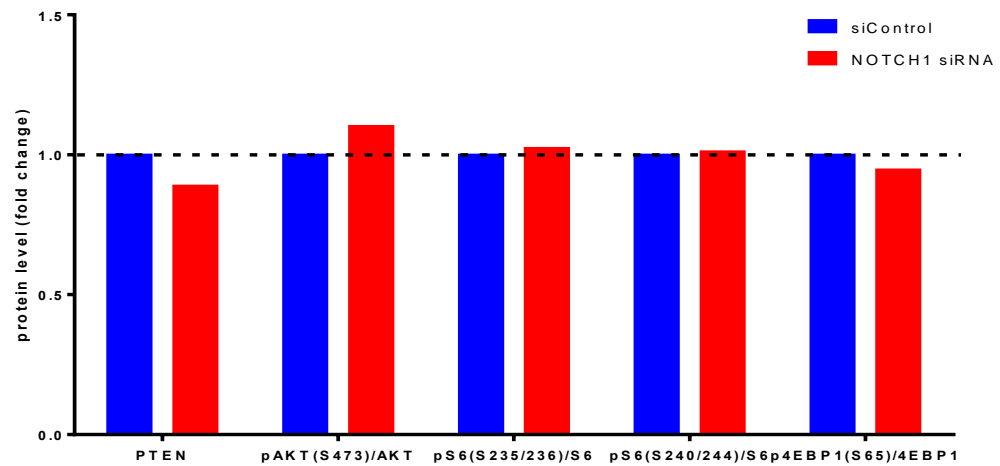


Figure 12: Silencing *NOTCH1* has no significant effect on the PI3K-PTEN-AKT/mTOR pathway.

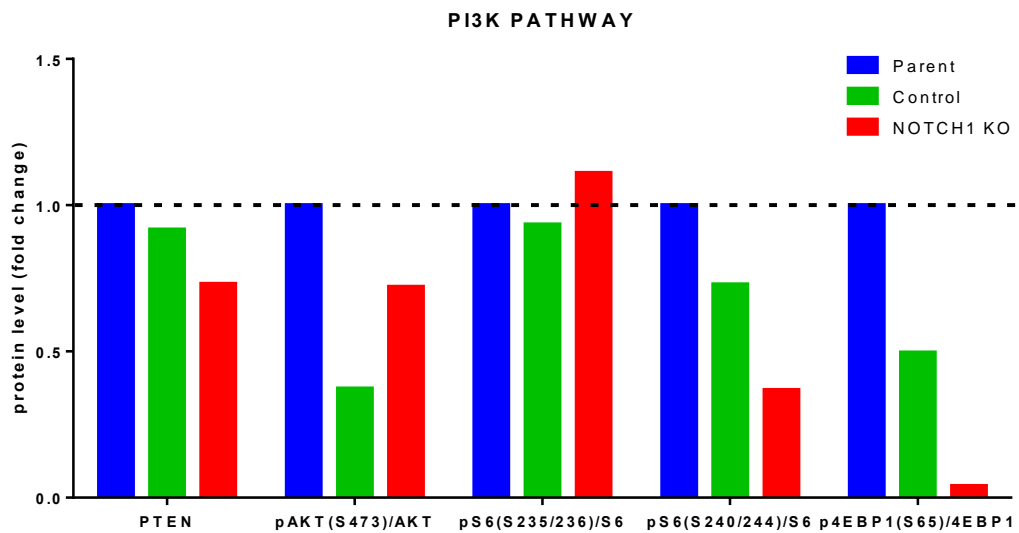
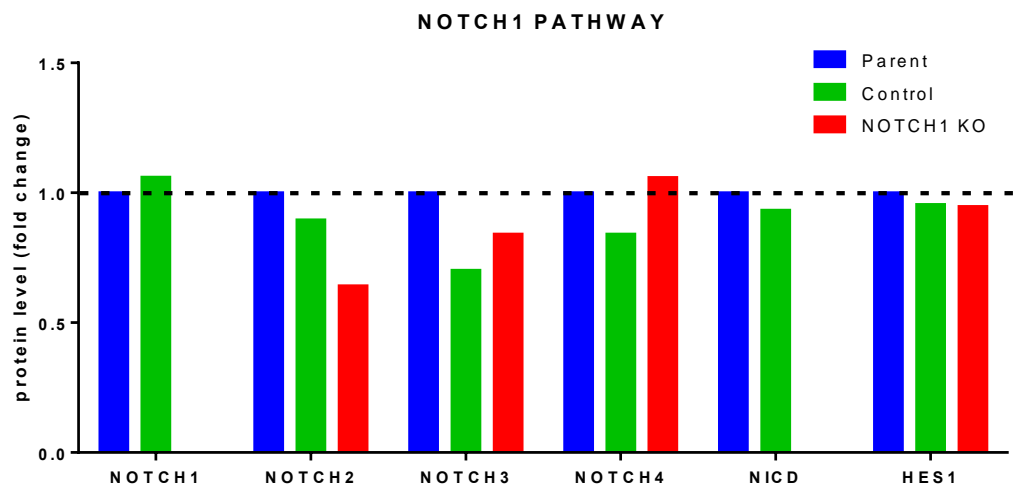
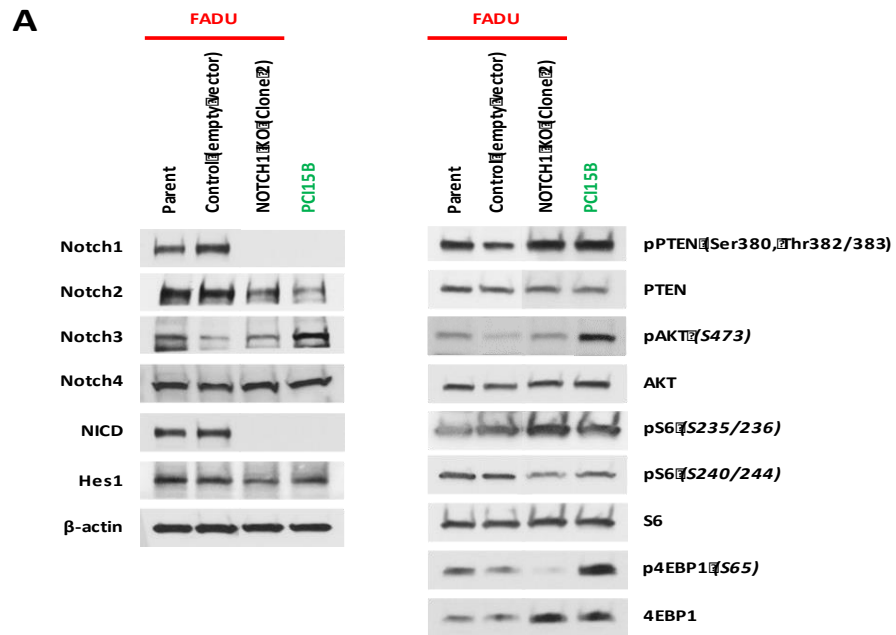
Western blot analysis and quantification of NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels after 20nM siRNA treatment for 24 hours in [A] FADU, [B] PJ34, and [C] OSC19. Control and treated samples are denoted in blue and red, respectively.

3.6 Knockout (KO) of *NOTCH1* leads to differential effects on the PI3K-PTEN-AKT/mTOR pathway

Using siRNA to silence the *NOTCH1* gene successfully inhibited NOTCH1 signaling, but did not have a significant effect on HES1 or the PI3K-PTEN-AKT/mTOR pathway. We decided to use a more specific genetic approach to prevent NOTCH1 signaling. We received FADU and PJ34 Crispr/Cas9-generated NOTCH1 KO cell lines from Dr. Mitchell Frederick's laboratory. Because both alleles of *NOTCH1* are stably deleted from both cell lines, we resolved this method of blocking NOTCH1 signaling to be the most efficient method of inhibition. We cultured the respective cell lines for 24 hours, collected lysate, and measured the NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway proteins via western blot analysis. Cells in the control group were transfected with an empty vector. Experiments were repeated twice in each cell line for validity and reliability measures (n=2).

We proposed that genetically deleting both alleles of *NOTCH1* would effectively prevent NOTCH1 signaling, leading to markedly increased PTEN activity and the subsequent inactivation of the PI3K-PTEN-AKT/mTOR pathway. Strikingly, the results were cell-line-specific. While both Crispr/Cas9-generated NOTCH1 KO cell lines successfully inhibited NOTCH1 signaling, as confirmed by the absence of NOTCH1 and NICD protein, the effect on the PI3K-PTEN-AKT/mTOR pathway was different in each cell line. Although the NOTCH1 KO treatment led to inactivation of the PI3K-PTEN-AKT/mTOR pathway in FADU, it led to activation of the pathway in PJ34 (Figure 13). The results are irrespective of the HES1 and PTEN protein levels, which were not affected by the NOTCH1 KO treatment, in each cell line. The data further confirms the observation that the NOTCH1-HES1-PTEN interaction in T-ALL may be weak or nonexistent in

HNSCC. The data also suggests that if there is a link between the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways in HNSCC, it may vary across the cell lines.



B

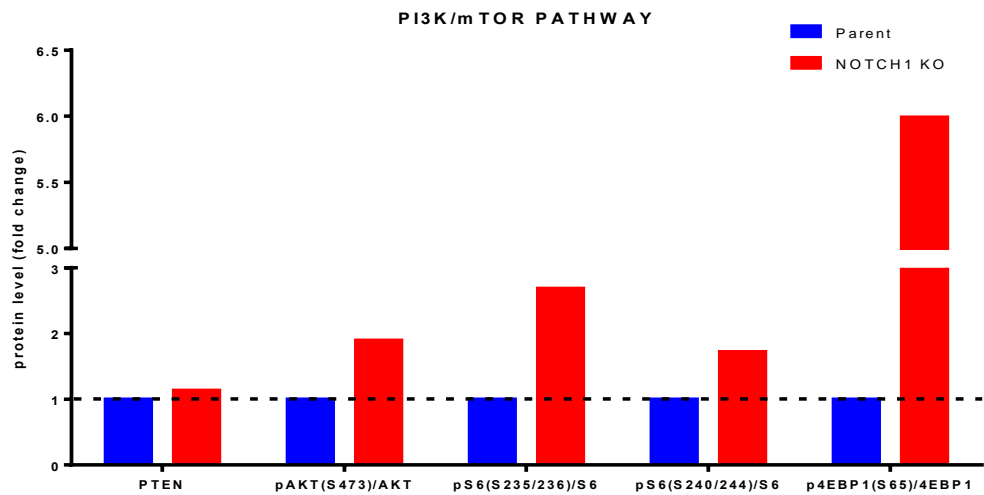
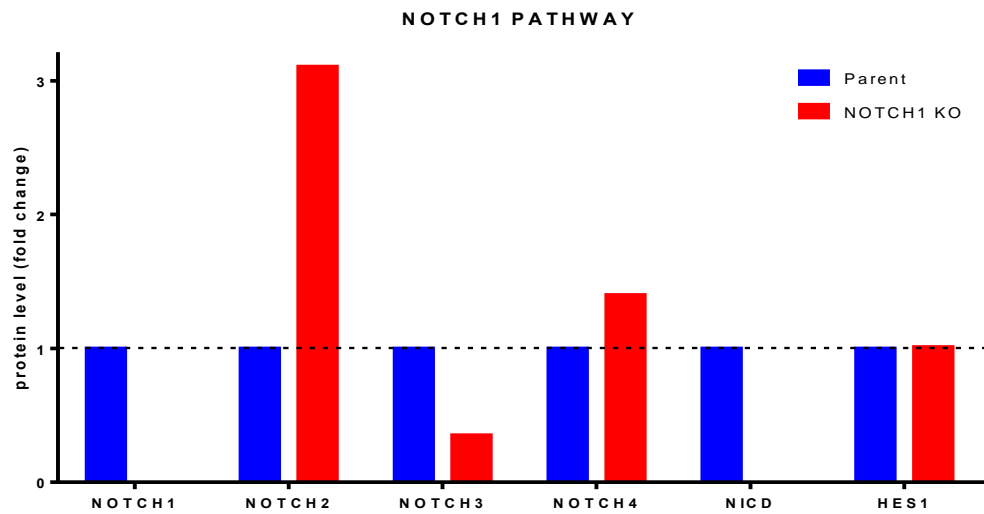
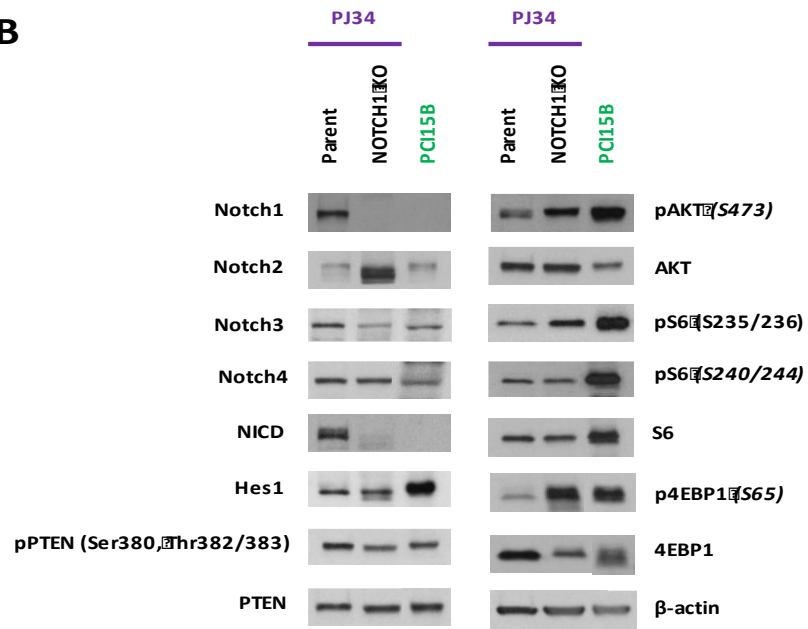


Figure 13: *NOTCH1*^{-/-} leads to differential effects on the PI3K-PTEN-AKT/mTOR pathway.

Western blot analysis and quantification of NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels in [A] FADU and [B] PJ34 Crispr/Cas9-generated NOTCH1 KO cell lines. Parental, control, and NOTCH1 KO samples are indicated by blue, green, and red, respectively.

3.7 Knockout (KO) of *NOTCH1* leads to differential effects on *HES1* and *PTEN* transcription in HNSCC

The Crispr/Cas9-generated deletion of both alleles of *NOTCH1* proved to be the most effective method of inhibiting NOTCH1 signaling. As a result, we sought to explore the effect of this method of inhibition on the transcription of *HES1* and *PTEN* in HNSCC. We cultured FADU and PJ34 Crispr/Cas9-generated NOTCH1 KO cell lines for 24 hours, isolated RNA, and measured *NOTCH1*, *HES1*, and *PTEN* mRNA levels via qRT-PCR. Cells in the control group were transfected with an empty vector. Experiments were repeated twice in each cell line for validity and reliability measures (n=2).

Based on the well-known NOTCH1-HES-PTEN association in T-ALL, we postulated that the Crispr/Cas9-mediated inhibition of NOTCH1 signaling would significantly decrease *HES1* transcription and greatly increase *PTEN* transcription. The findings displayed in Figure 14 show that Crispr/Cas9-mediated inhibition of NOTCH1 signaling significantly downregulates *HES1* transcription but has no significant effect on *PTEN* transcription in both cell lines. The data is promising because it supports the premise of the central hypothesis, which holds that *HES1* transcription is regulated by the NOTCH1 pathway. Additionally, the results of the western blot and qRT-PCR analyses in the Crispr/Cas9-generated NOTCH1 KO cell lines are the only evidence that an interaction between the NOTCH1 pathway and the PI3K-PTEN-AKTmTOR pathway exists in HNSCC. Although the mediators of communication between the pathways in HNSCC may differ from those in T-ALL, the data suggests that an interaction between the pathways is present in HNSCC.

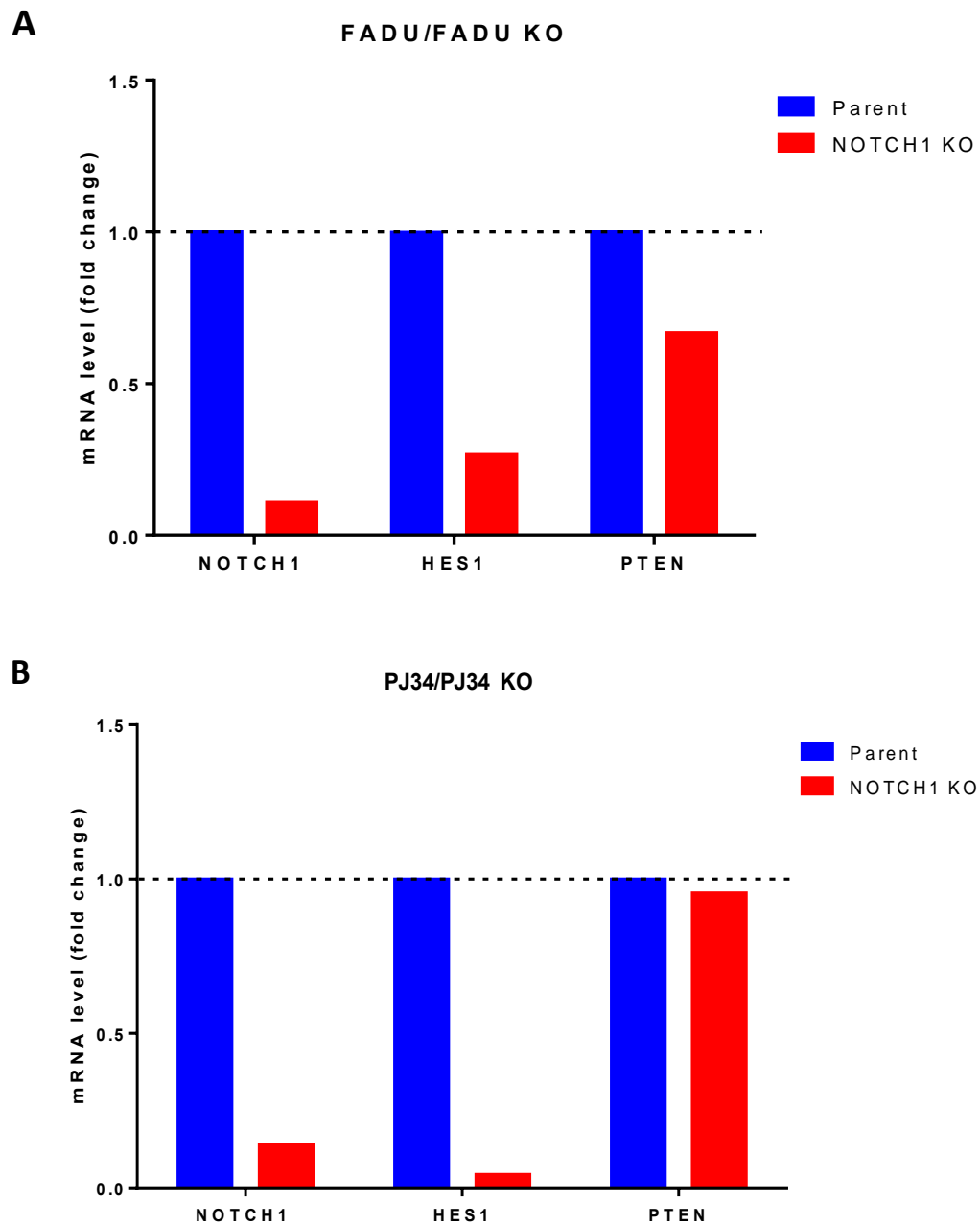


Figure 14: *NOTCH1*^{-/-} has differential effects on *HES1* and *PTEN* transcription in HNSCC.

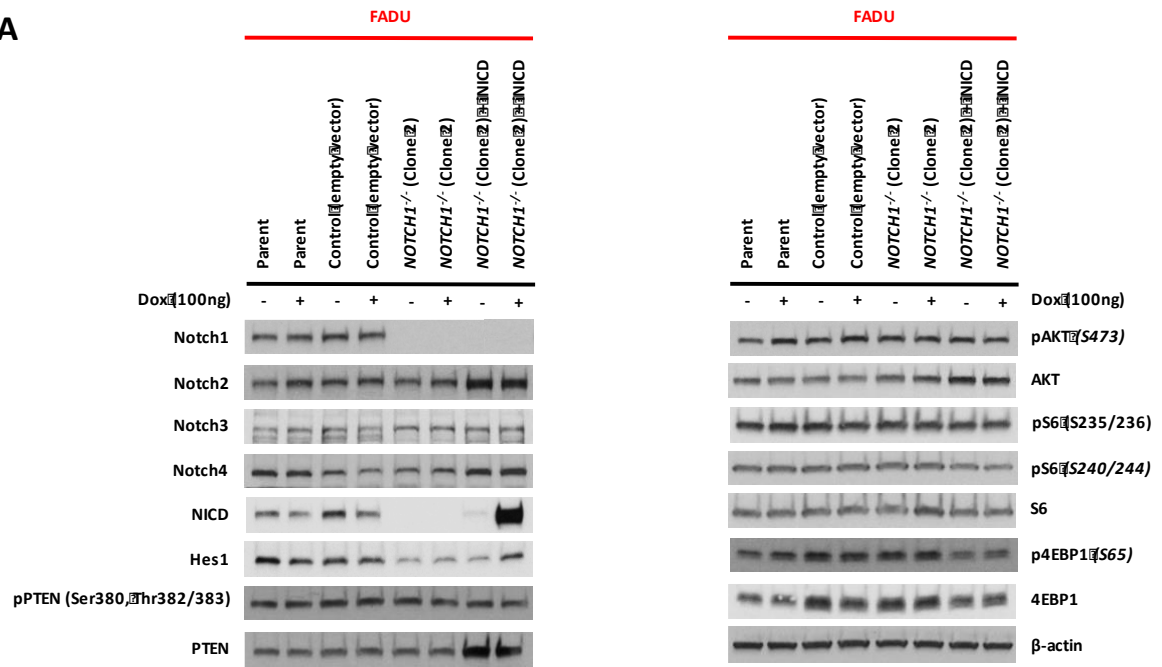
Quantification of *NOTCH1*, *HES1*, and *PTEN* mRNA levels after qRT-PCR analysis of [A] FADU and [B] PJ34 Crispr/Cas9-generated *NOTCH1* KO cell lines. Parental and *NOTCH1* KO samples are indicated by blue and red, respectively.

3.8 Restoration of NOTCH1 signaling has no significant effect on the PI3K-PTEN-AKT/mTOR pathway in HNSCC

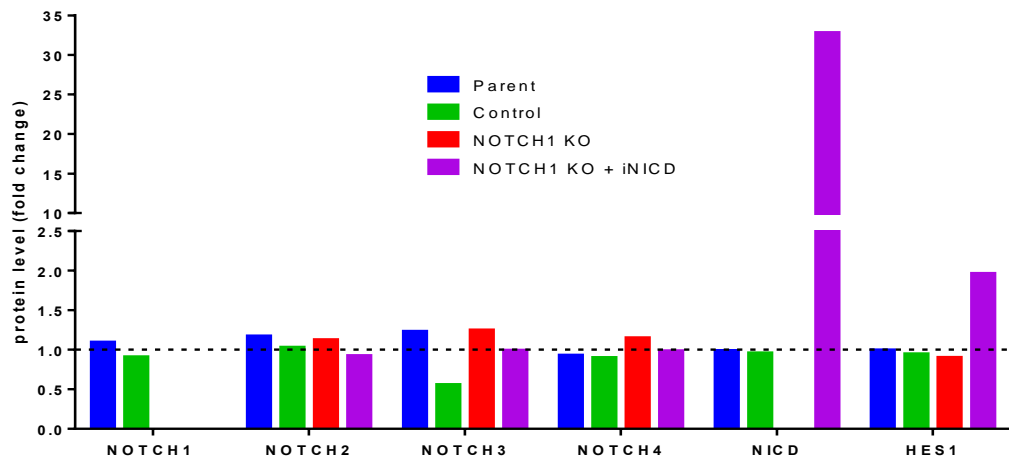
Because the results of the western blot and qRT-PCR analyses in the Crispr/Cas9-generated NOTCH1 KO cell lines were in accordance with our central hypothesis, we sought to validate the findings by restoring active NOTCH1 signaling in the NOTCH1 KO cell lines. Dr. Mitchell Frederick's laboratory also developed FADU and PJ34 Crispr/Cas9-generated NOTCH1 KO cell lines that expressed a doxycycline-inducible Notch1 intracellular domain (NICD) system. Simply put, the inducible NICD system allowed us to restore active NOTCH1 signaling in the NOTCH1 KO cell lines, which are entirely deficient of any NOTCH1 signaling capabilities. We treated both cell lines with 100ng/ml of doxycycline for 24 hours. We then collected lysate and measured NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels via western blot analysis. Experiments were repeated twice in each cell line for validity and reliability measures (n=2).

We expected that restoring active NOTCH1 signaling in the NOTCH1 KO cell lines would yield an effect on the PI3K-PTEN-AKT/mTOR pathway that was the opposite of what was exhibited in the Crispr/Cas9-mediated inhibition of NOTCH1 signaling. Restoring NOTCH1 signaling would upregulate *HES1* transcription and downregulate PTEN activity, resulting in the hyper-activation of the PI3K/AKT/mTOR pathway. However, restoring NOTCH1 signaling had no significant effect on HES1 or the PI3K-PTEN-AKT/mTOR pathway in either cell line (Figure 15). There were no consistent or significant differences among the pathway proteins in the control and treated samples of each of the cell lines. The data offers a strong rationale for moving further along the NOTCH1 pathway and focusing on the manipulation of HES1 to investigate the interaction between the NOTCH1 and the PI3K-PTEN-AKT/mTOR pathways.

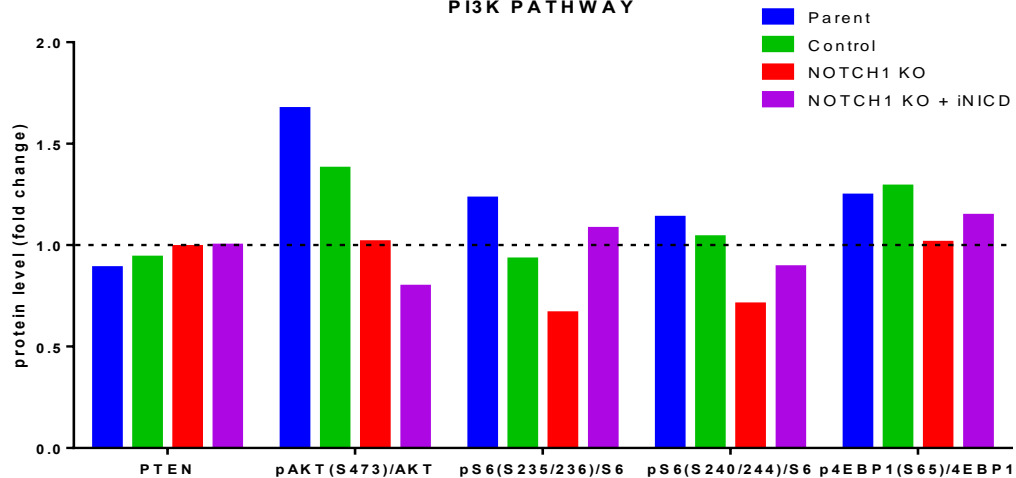
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NOTCH1 PATHWAY



PI3K PATHWAY



B

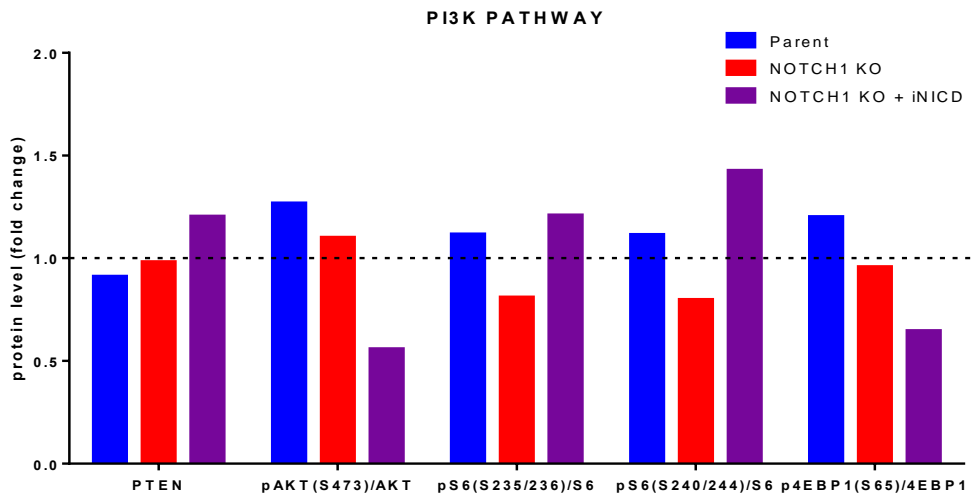
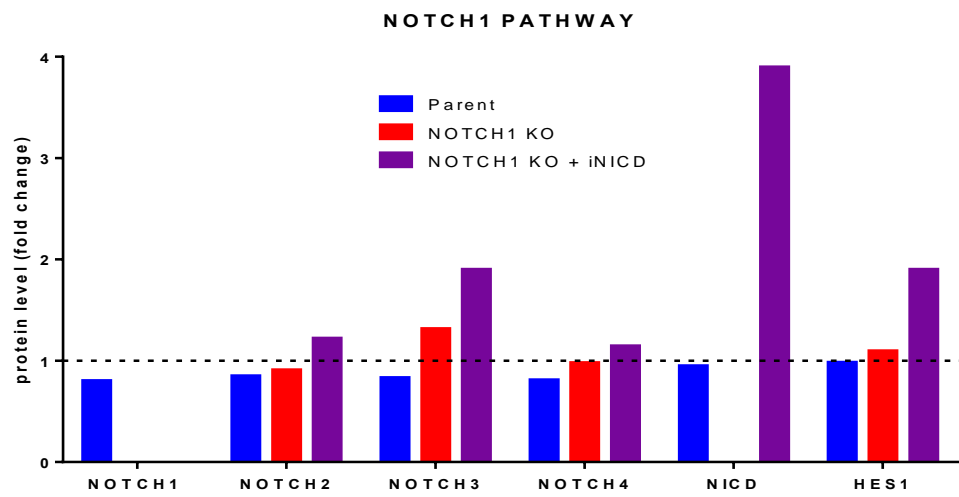
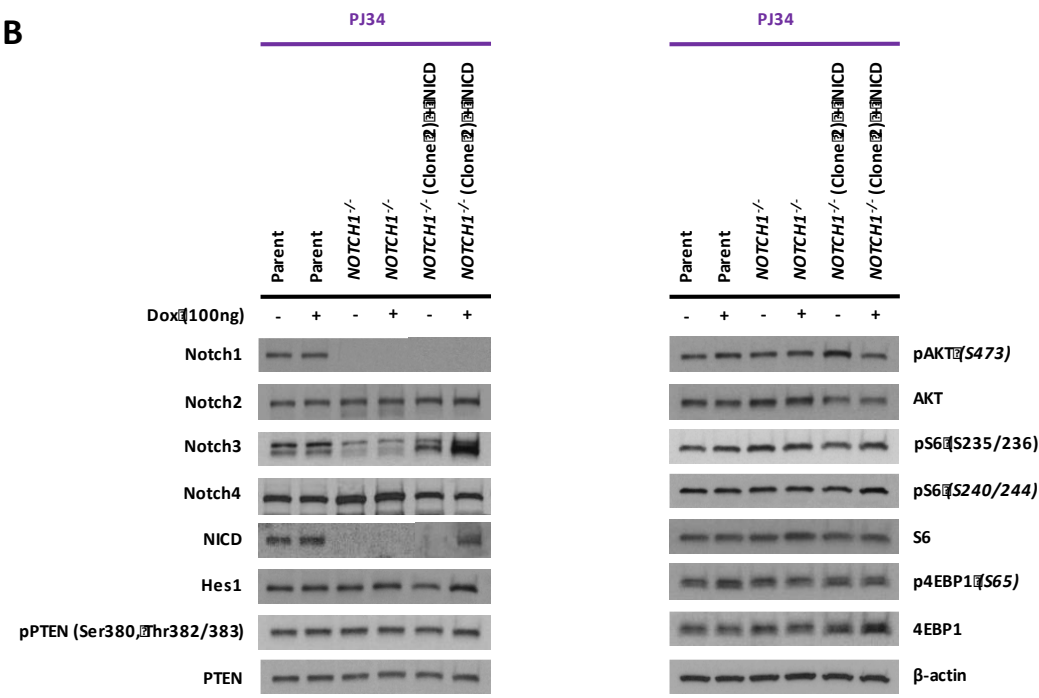


Figure 15: Restoring NOTCH1 signaling has no significant effect on the PI3K-PTEN-AKT/mTOR pathway in HNSCC.

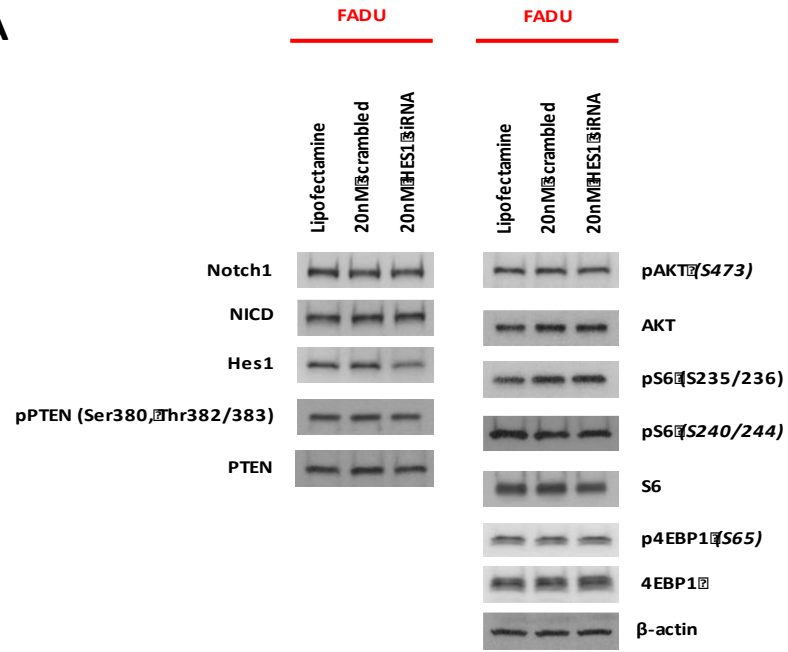
Western blot analysis and quantification of NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels in [A] FADU and [B] PJ34 Crispr/Cas9-generated NOTCH1 KO cell lines transfected with the inducible NICD vector. Parental, control, NOTCH1 KO, and NOTCH1 KO + iNICD samples are indicated by blue, green, red, and purple, respectively.

3.9 Knock down (KD) of *HES1* has no significant effect on the PI3K-PTEN-AKT/mTOR pathway in HNSCC

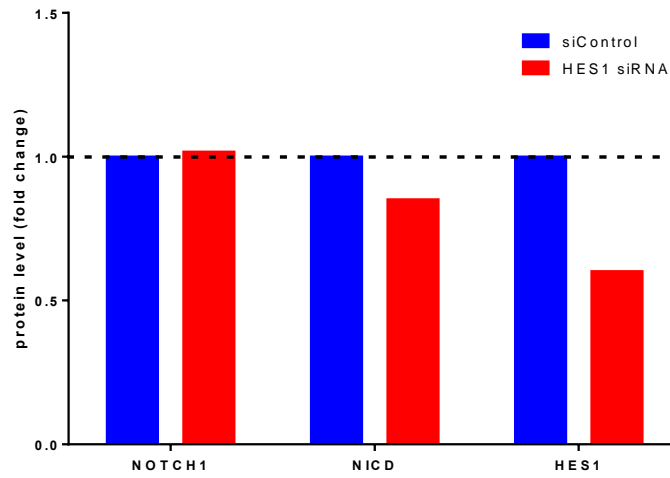
According to the outcomes of previous experiments, we have concluded that manipulating the NOTCH1 pathway does not have a significant or consistent effect on the PI3K-PTEN-AKT/mTOR pathway in HNSCC. However, manipulating the pathway has a minimal effect on HES1 protein levels in HNSCC. We postulate that manipulating HES1 may affect PTEN transcription because HES1 is a more direct regulator of PTEN and ultimately PI3K/AKT/mTOR pathway activity. We treated the 3 *NOTCH1* wild-type HNSCC cell lines (FADU, PJ34, and OSC19) with 20nM siRNA targeting the *HES1* gene for 24 hours. HES1 protein and mRNA have a half-life of approximately 20 minutes, making 24 hours a suitable time point for silencing the *HES1* gene (Kobayashi et al., 2015). The control groups of the respective cell lines were treated with 20nM non-targeting siRNA for 24 hours. We then collected lysate and measured NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels via western blot analysis. Experiments were repeated twice in each cell line for validity and reliability measures (n=2).

Figure 16 reveals that silencing the *HES1* gene has no significant effect on PTEN or the PI3K/AKT/mTOR pathway in either of the cell lines. Taken together with the experiments involved in manipulating NOTCH1 signaling, the data supports the observation that the NOTCH1 pathway does not affect the PI3K-PTEN-AKT/mTOR pathway in HNSCC. Given the unestablished NOTCH-HES1-PTEN association in HNSCC, we should explore other potential mediators of communication between the NOTCH1 pathway and the PI3K-PTEN-AKT/mTOR pathway in HNSCC (i.e. PTEN or AKT).

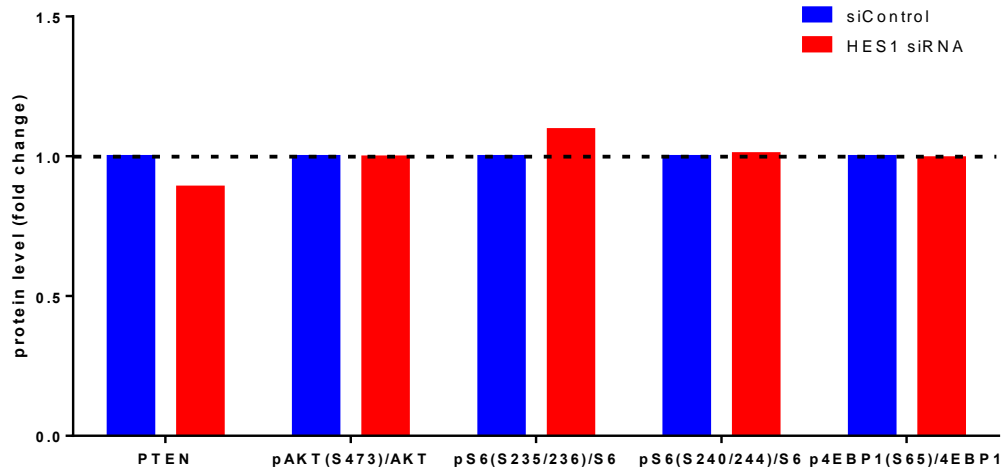
A



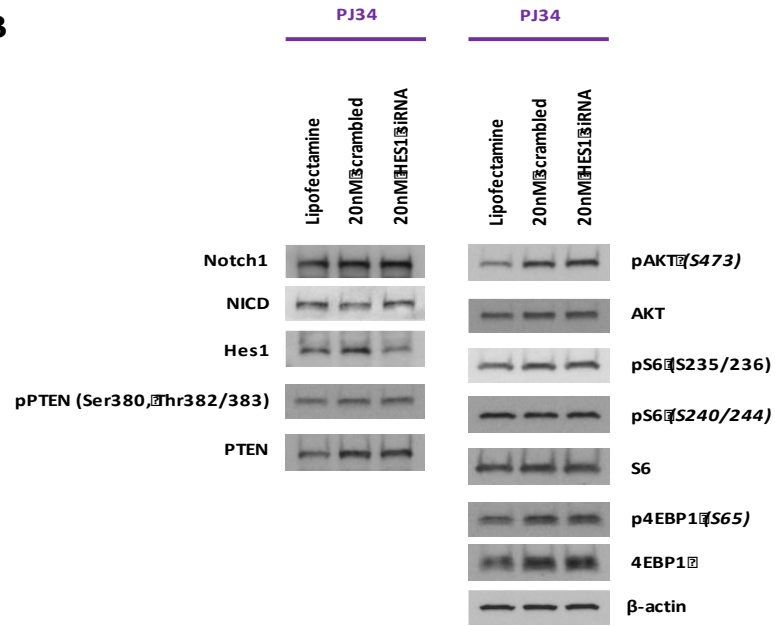
NOTCH1 PATHWAY



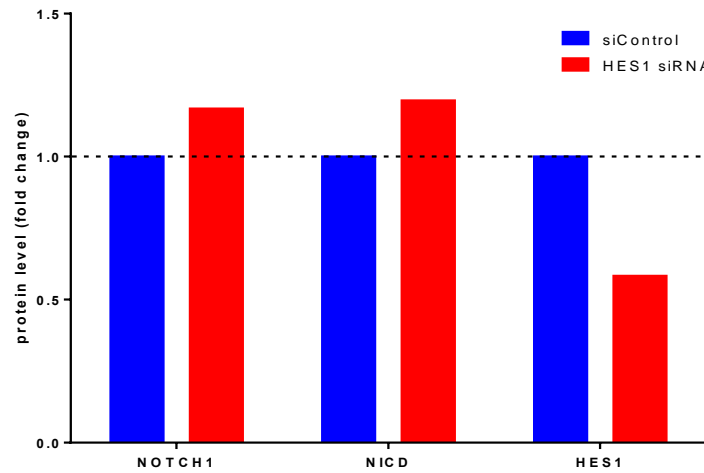
PI3K PATHWAY



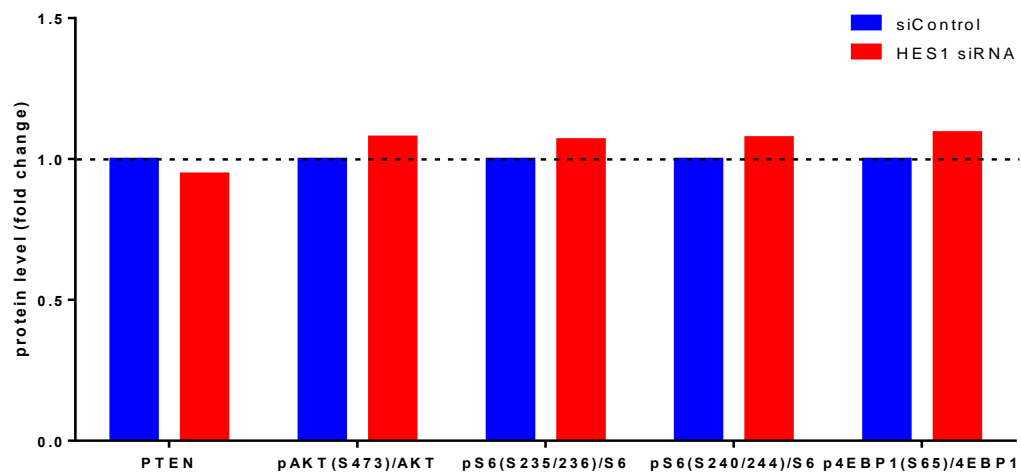
B



NOTCH1 PATHWAY



PI3K PATHWAY



C

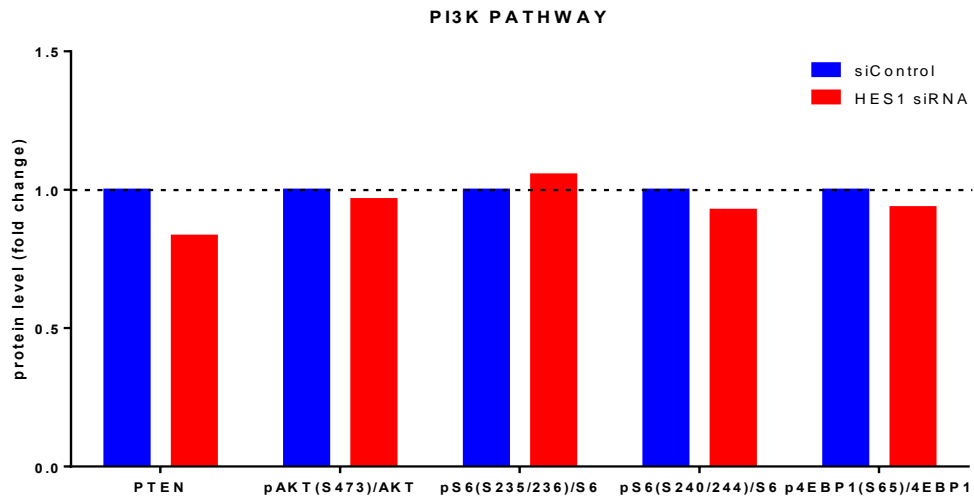
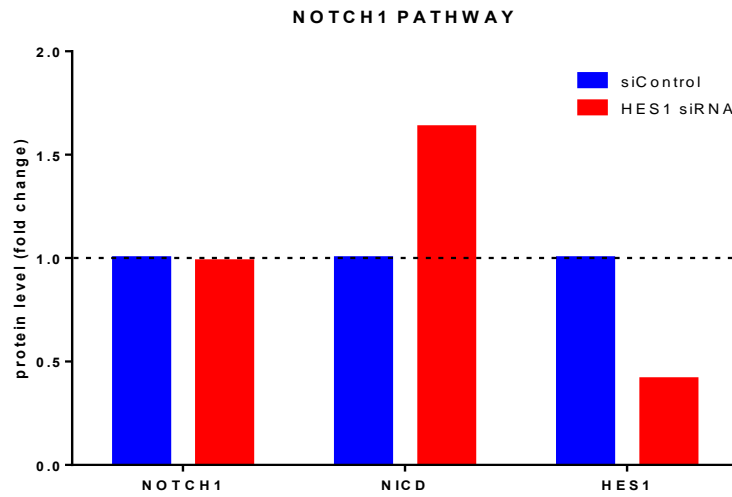
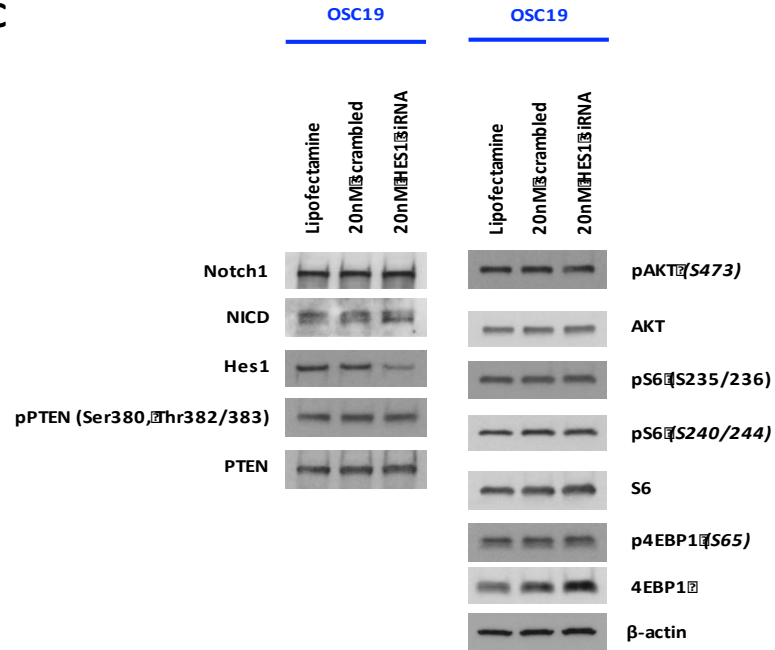


Figure 16: Silencing *HES1* has no significant effect on the PI3K-PTEN-AKT/mTOR pathway.

Western blot analysis and quantification of NOTCH1 pathway and PI3K-PTEN-AKT/mTOR pathway protein levels after 20nM siRNA treatment for 24 hours in [A] FADU, [B] PJ34, and [C] OSC19. Control and treated samples are denoted in blue and red, respectively.

CHAPTER 4:

DISCUSSION & FUTURE DIRECTIONS

4.1 Discussion

While the oncogenic role of NOTCH1 is well-established in T-ALL, the tumor suppressive role of NOTCH1 in HNSCC is poorly understood. Preliminary studies in our laboratory report that *NOTCH1* mutant HNSCC cell lines are sensitive to dual PI3K/mTOR inhibitors, suggesting a potential interaction between the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways *in vivo*. In T-ALL, the crosstalk between both pathways has been heavily studied and is distinctively characterized in the literature. In HNSCC, on the other hand, the interaction between both pathways is largely unknown and remains the focus of most prominent research in the field. Although the findings of many of the experiments in this research consist of negative data, we have been able to formulate rather insightful conclusions regarding the interaction between the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways in HNSCC.

We employed three methods of inhibition to block NOTCH1 signaling in *NOTCH1* wild-type HNSCC cell lines, with each successive method being more specific in its targeting potential than its predecessor. Using OMP-52M51 to block NOTCH1 signaling had no significant effect on HES1 or the PI3K-PTEN-AKT/mTOR pathway at the protein level. Additionally, silencing the *NOTCH1* gene to block NOTCH1 signaling had no significant effect on HES1 or the PI3K-PTEN-AKT/mTOR pathway at the protein level. The findings of these experiments suggest the nonexistence of any type of interaction between the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways in HNSCC.

On the other hand, employing a more specific genetic method to prevent NOTCH1 signaling yielded more promising results. The Crispr/Cas9-mediated inhibition of NOTCH1 signaling led to downregulated PI3K/AKT/mTOR activity in FADU and upregulated PI3K/AKT/mTOR activity in PJ34. The findings restore our confidence in the initial observation that a possible interaction exists between the NOTCH1 and PI3K-

PTEN-AKT/mTOR pathways in HNSCC. Nevertheless, the cell-line-specific findings show that the pathways may be regulated differently across the cell lines. The findings further suggest that the active domains of the NOTCH1 receptor must be completely removed from the cell to effectively inhibit its signaling and observe any effects that pathway inhibition may have on other pathways in the cell. Because the treatment had no significant effect on the HES1 and PTEN protein levels of both cell lines, we can infer that the potential crosstalk between both pathways is not mediated by either HES1 or PTEN.

Restoring NOTCH1 signaling in *NOTCH1* wild-type HNSCC cell lines had no significant effect on HES1 or the PI3K-PTEN-AKT/mTOR pathway at the protein level, imparting a fundamental indication that the link between both pathways may be a rather weak link. However, qRT-PCR analysis following each method of inhibiting NOTCH1 signaling revealed that *HES1* mRNA levels were significantly reduced after blocking NOTCH1 signaling, but there was no effect on the *PTEN* mRNA levels. The findings confirm that the NOTCH1-HES1 association present in T-ALL is also present in HNSCC. Inactivating NOTCH1 signaling inhibits *HES1* transcription, resulting in significantly decreased *HES1* mRNA levels. The results also suggest that the well-established interaction between HES1 and PTEN in T-ALL is not present in HNSCC. Additionally, silencing *HES1* had no significant effect on PTEN or the PI3K/AKT/mTOR pathway at the protein level, providing sufficient evidence that the link between HES1 and PTEN does not exist in HNSCC. Taken together, the results of the experiments in this research support the observation that an interaction does not exist between the NOTCH1 pathway and the PI3K-PTEN-AKT/mTOR in HNSCC.

4.2 Future Directions

The findings of this project provide valuable insight into understanding whether the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways communicate in HNSCC. The data also imparts fundamental objectives for directing experimental efforts to identify potential mediators of communication between both pathways. Establishing a distinct relationship between NOTCH1, HES1 and PTEN in HNSCC cell lines is crucial to understanding how the NOTCH1 and PI3K-PTEN-AKT/mTOR pathways communicate in HNSCC. Unfortunately, the experimental efforts executed in this project did not succeed in identifying the mechanism of crosstalk between both pathways.

Employing more specific genetic methods to manipulate target proteins will enable us to study the effects of the respective treatments on pathway interactions. For instance, utilizing Crispr/Cas9 methodologies to genetically delete both alleles of *HES1* from the cells may enable us to identify an association between HES1 and PTEN in HNSCC. We will also consider the possibility of bidirectional crosstalk between the pathways in HNSCC. To test our hypothesis, we will employ chemical and genetic methodologies to manipulate each component of the PI3K-PTEN-AKT/mTOR pathway and measure the effect the treatment has on the NOTCH1 pathway. Additionally, utilizing high throughput, quantitative, functional proteomic and genomic technologies, such as reverse phase protein array (RPPA) and RNA sequencing, will aid us in narrowing our search for more specific mediators of communication between both pathways. Elucidating the potential mechanisms of communication between the NOTCH1 and the PI3K-PTEN-AKT/mTOR pathways presents both pathways as fundamental targeting agents for improving many HNSCC targeted therapies.

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Vita

Kyriante' Savonn Henry was born in New Orleans, Louisiana on April 16, 1993. She is the daughter of Kione Natasha Duncan and Louis Anthony Henry. She received her academic diploma from Xavier University Preparatory High School in New Orleans, Louisiana in May 2011. She attended Xavier University of Louisiana in New Orleans, Louisiana, where she received the degree of Bachelor of Science with a major in Psychology and a minor in Chemistry in May 2015. In August of 2015, she entered The University of Texas MD Anderson Cancer Center UTHHealth Graduate School of Biomedical Sciences, aspiring to attain the degree of Master of Science in Biomedical Sciences with a concentration in Cancer Biology.

Permanent address:
4911 Cernay Street
New Orleans, Louisiana 70129